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**Let's Get Cracking —Nut Allergy Diagnostics
and Peanut Oral Immunotherapy**

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LET'S GET CRACKING —NUT ALLERGY DIAGNOSTICS AND PEANUT ORAL IMMUNOTHERAPY

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ACADEMIC DISSERTATION

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ABSTRACT

Background: Nut allergy diagnostics is complicated, due to asymptomatic sensitization and pollen-induced cross-sensitization. In addition, it is usually a life-long disease, and traditional treatment involves the avoidance of nuts and the administration of emergency medication in accidental exposures.

Aims: To study nut allergy diagnostics by evaluating associations with nut- and birch pollen sensitizations and the performance of IgE microarray in peanut allergy diagnostics. In addition, to study the efficacy and safety of oral peanut immunotherapy and how the treatment modifies antibody profiles.

Methods: We analyzed nut- and birch pollen sensitizations in a register of over 100,000 subjects from southern and northern Finland. We studied IgE microarray and avoidance diets in 102 patients who underwent a peanut challenge. In order to assess immunotherapy efficacy and antibody changes, we conducted an intervention study in which 39 patients received peanuts orally with increasing doses, and 21 patients served as controls.

Results: Nut sensitizations associated strongly with birch pollen sensitization in both southern and northern Finland, and in this regard hazelnut, peanut, and almond sensitizations exhibited the strongest links. Up to 84% of hazelnut-sensitized patients had simultaneous sensitization to birch pollen, while the majority of sensitized subjects reported no or only mild oral symptoms from exposure to nuts. In the microarray, Ara h 2 and Ara h 6 were the most accurate allergens in discriminating between peanut allergy and tolerance. Commonly, peanut-sensitized patients avoided several nut species, but in the species-specific tests, sensitization to other nuts was infrequent. In immunotherapy, 33 (85%) of 39 patients achieved the target dose of 800 mg peanut protein (approximately four peanut kernels), and specific IgG4 increased strongly. No neosensitizations emerged in the microarray screening, but IgE levels decreased for the most important peanut allergens, Ara h 2 and Ara h 6.

Conclusions: The impact of birch pollen sensitization on nut sensitizations is remarkable in Finland, and so it should be taken into account in nut allergy diagnostics. Based on species-specific allergen tests, patients can introduce several previously avoided nuts into their diet. Peanut oral immunotherapy is effective in desensitizing severely allergic children and adolescents. No neosensitizations develop and other sensitizations are unaffected, which shows that peanut oral immunotherapy is highly allergen-specific.

TIIVISTELMÄ

Tausta: Pähkinäallergian diagnostiikka on haastavaa oireettoman herkistymisen ja siitepölyjen aiheuttaman ristiherkistymisen vuoksi. Pähkinäallergia on yleensä elinikäinen, ja perinteisenä hoitona on vain pähkinöiden välttäminen ja ensiapulääkkeet vahinkoaltistustilanteissa.

Tavoitteet: Tutkia pähkinäallergian diagnostiikkaa selvittämällä pähkinä- ja koivuherkistymisten yhteyksiä ja IgE-mikrosirun toimivuutta maapähkinäallergisilla potilailla sekä tutkia maapähkinäallergian siedätyshoidon tehokkuutta ja hoidon aiheuttamia muutoksia vasta-aineprofiileissa.

Menetelmät: Pähkinä- ja koivuherkistymisiä selvitettiin yli 100 000 potilaan aineistossa Etelä- ja Pohjois-Suomessa. Maapähkinälle altistettujen potilaiden (n=102) aineistossa analysoitiin IgE-mikrosiruprofiilit ja kyselytutkimuksella arvioitiin eri pähkinälajien käyttöä. Siedätyshoitoa ja sen aiheuttamia vasta-ainemuutoksia tutkittiin interventioasetelmassa, jossa 39 potilasta sai maapähkinää suun kautta hitaasti nousevin annoksin ja 21 yhtä vakavasti maapähkinäallergista potilasta toimi verrokkiryhmänä.

Tulokset: Pähkinä- ja koivuherkistymiset olivat vahvasti yhteydessä toisiinsa sekä Etelä- että Pohjois-Suomessa. Voimakkaimmin koivuherkistymiseen olivat yhteydessä hasselpähkinä-, maapähkinä- ja manteliherkistymiset.

Hasselpähkinäherkistyneistä jopa 84 % oli samanaikaisesti herkistynyt koivun siitepölylle. Suurin osa pähkinäherkistyneistä ilmoitti sietävänsä pähkinöitä täysin oireitta tai ilmoitti saavansa ainoastaan lieviä suuoireita. Mikrosiruissa maapähkinän allergeenit Ara h 2 ja Ara h 6 erottelivat parhaiten vakavasti allergiset. Potilaista suuri osa ilmoitti välttävänsä useita pähkinälajeja, mutta siruanalyysissä vain harvalla nähtiin IgE-vasteita lajikohtaisille allergeeneille. Siedätyshoidossa tavoiteannoksen 800 mg maapähkinäproteiinia (noin neljä maapähkinää) saavutti 33/39 (85 %) potilasta. Siedätysryhmässä spesifinen IgG4 nousi voimakkaasti. IgE-mikrosiruprofiileissa uusia herkistymisiä ei kehittynyt. Aikaisemmissa herkistymisissä ei tapahtunut muutoksia lukuun ottamatta allergeeneja Ara h 2 ja Ara h 6, joiden vasta-ainetaso laski.

Johtopäätökset: Suomessa koivuherkistymisen vaikutus pähkinäherkistymiseen on huomattava. Tämä tulee ottaa huomioon pähkinäallergian diagnostiikassa herkistymisiä arvioitaessa. Suuri osa maapähkinäherkistyneistä potilaista välttää useita muitakin pähkinälajeja, mutta lajikohtaisten allergeenitutkimusten perusteella monien pähkinälajien välttö on tarpeetonta.

Maapähkinäsiedätys on tehokas hoito nostamaan oirekynnystä maapähkinäallergisilla. Siedätys ei aiheuta uusia tai vahvasta olemassa olevia herkistymisiä, mikä on tärkeää hoidon turvallisuuden kannalta.

ABBREVIATIONS

CI Confidence interval

DBPCFC Double-blind placebo-controlled food challenge

FeNO Fractional exhaled nitric oxide

FEV1 Forced expiratory volume in one second

IgA Immunoglobulin A

IgE Immunoglobulin E

IgG Immunoglobulin G

IgM Immunoglobulin M

IL Interleukin

ISAC Immuno Solid-phase Allergen Chip

ISU-E ISAC standardized units for IgE

LOAELs Lowest observed adverse effect levels

LTP Lipid transfer protein

NOAELs No observed adverse effect levels

OAS Oral allergy syndrome

OFC Oral food challenge

OIT Oral immunotherapy

PD20FEV1 The cumulative dose of methacholine provoking a 20% decline in forced expiratory volume in one second

PFS Pollen food syndrome

PPV Positive predictive value

PR-10 Pathogenesis-related group 10

PRACTALL Practical Allergy Report

RCT Randomized controlled trial

RR Risk ratio

SLIT Sublingual immunotherapy

SPT Skin prick test

SU Sustained unresponsiveness

TGF Transforming growth factor

TNO FARRP the Netherlands Organisation for applied scientific research Food Allergy Research and Resource Program

LIST OF ORIGINAL PUBLICATIONS

- I. Uotila R, Kukkonen AK, Pelkonen AS, Mäkelä MJ.
Cross-sensitization profiles of edible nuts in a birch-endemic area.
Allergy 71:514-21. 2016.
- II. Kukkonen AK, Uotila R, Malmberg LP, Pelkonen AS, Mäkelä MJ.
Double-blind placebo-controlled challenge showed that peanut oral immunotherapy was effective for severe allergy without negative effects on airway inflammation.
Acta paediatrica 106: 274–281. 2017.
- III. Uotila R, Kukkonen AK, Greco D, Pelkonen AS, Mäkelä MJ.
Peanut oral immunotherapy decreases IgE to Ara h 2 and Ara h 6 but does not enhance sensitization to cross-reactive allergens.
The Journal of Allergy Clinical Immunology 139: 1393–1396. 2017.
- IV. Uotila R, Kukkonen AK, Blom WM, Remington B, Westerhout J, Pelkonen AS, Mäkelä MJ.
Component-resolved diagnostics demonstrates that most peanut-allergic individuals could potentially introduce tree nuts to their diet.
Clinical and Experimental Allergy 48:712–721. 2018.

The publications above are referred to in the text by their Roman numerals.

1. INTRODUCTION

Food-induced allergic reactions are an important health burden especially in children and adolescents. Eight foods (peanut, tree nuts, egg, milk, fish, shellfish, wheat, and soy) cause most food allergic reactions, and nuts are the most common culprit in regard to food-induced severe allergic reactions.

Edible nuts are a heterogeneous group including species that in a botanical sense are not what one may refer to as ‘true’ nuts. In the allergological literature, a common classification is to separate peanut and tree nuts. Peanut, also called a groundnut, is a legume similar to soybean and grows on the ground. Tree nuts comprise almond, beech nut, Brazil nut, butternut, cashew, chestnut, chinquapin, coconut, hazelnut, ginkgo nut, hickory nut, lichee nut, macadamia nut, pecan, pine nut, pili nut, pistachio, shea nut, and walnut—according to the U.S. Food and Drug Administration.¹

Plant-derived foods are especially interesting from an allergological point of view, due to the phenomenon of cross-reactivity. Plant pollens as aeroallergens can sensitize via the respiratory tract, but cross-reactive foods cause symptoms when ingested. Some individuals experience only mild allergic reactions from nuts, whereas others have severe and even life-threatening symptoms. It is therefore important to separate mild allergies from severe reactions.

Unnecessary avoidance diets are a burden, but severely allergic patients still need vigilance and the right patient advice.

In allergic sensitization, the human body produces IgE antibodies against a normal antigen, for example food. Measuring IgE is a major part of allergy diagnostics, but presence of IgE does not equate to a clinical allergy, as asymptomatic sensitization is common. The food challenge is the gold standard of food allergy diagnostics, but it poses the risk of severe reaction and is laborious, and therefore other diagnostic methods are necessary.

In modern allergology, molecule-specific IgE, instead of the whole-allergen specific IgE, is measured. Molecular allergology has increased diagnostic accuracy markedly, as clinically relevant allergens instead of insignificant cross-reacting allergens can be selected for testing.

In the treatment of food allergies, the traditional method is to avoid the allergenic food and have emergency medications at hand in case of accidental exposure. Immunotherapy is an established treatment in severe pollen allergy, but immunotherapy for food allergy is currently in research. By increasing the threshold for allergic symptoms, the allergic individual will be safe from severe symptoms caused by accidental exposures to small amounts of the allergenic food. By starting from very low amounts of the allergenic food, and increasing these amounts slowly, it is possible to avoid symptoms and induce tolerance to the allergen. A complete cure to the allergy may currently not be possible, but symptom relief and lowering risk would be major benefits for severely allergic

patients and their families.

This study was undertaken to examine the diagnostics of nut allergy in an area where birch pollen exposure is high, and to examine the efficacy and safety of peanut oral immunotherapy and its effects on antibody profiles.

2. LITERATURE REVIEW

2.1 FOOD ALLERGY

The National Institute of Allergy and Infectious Diseases defines a food allergy as “an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food.”² The most common childhood food allergies are staple food allergies, caused by cow’s milk and hen’s egg.³ These allergies are usually outgrown, in contrast to nut and shellfish allergies, which tend to persist into adulthood. Food allergies are classified based on underlying immunological mechanisms. Most food allergies are IgE-mediated and result in rapid symptoms, while non-IgE-mediated food allergies are delayed in the onset of symptoms and significantly less common. Mixed-type (IgE- and cell-mediated) allergies include food-allergy-associated atopic dermatitis, eosinophilic esophagitis, and other eosinophilic gastrointestinal disorders.⁴ In IgE-mediated reactions mast cells and basophils release preformed inflammatory mediators which lead to an immediate reaction. Nut allergies are mostly IgE-mediated.

2.2 IMMUNOLOGY OF AN IGE-MEDIATED FOOD ALLERGY

Immunoglobulin E (IgE) was discovered in 1967.^{5, 6} The overall amount of IgE in the human body is low compared to other immunoglobulins, and it is needed to prevent helminth infections, however, the body also produces IgE in allergic sensitization. In addition to IgE, IgG4, which is also associated with allergies, is a member of the IgG antibody isotype that includes four subtypes (1-4), of which IgG1 is the most abundant. IgG4 is produced in response to chronic allergen exposure, and it has anti-inflammatory features. Importantly, it has been associated with tolerance to allergens.⁷

2.2.1 ORAL TOLERANCE AND SENSITIZATION VIA THE GASTROINTESTINAL TRACT

Not being allergic to a food requires an active process to take place, known as ‘oral tolerance’,⁸ but when this tolerance fails, allergic sensitization takes place.⁹ Antigens transfer from the intestinal lumen via the epithelial barrier, which after an antigen-presenting cell captures the antigen, migrates to the lymph node, and presents the antigen to a naive T cell. In oral tolerance, the naive T cell develops into a regulatory T cell on the basis of antigen and cytokine signaling. Retinoic acid, indoleamine 2,3-dioxygenase, and TGF-beta

provide critical signals for regulatory T cell development, and T regulatory cells suppress inflammatory reactions by producing cytokines such as TGF-beta and IL-10. When oral tolerance fails and sensitization occurs, the naive T cell develops into a T helper type 2 cell, which acts on B cells by IL-4 and IL-13, causing the B cells to switch from producing IgM to IgE.¹⁰

2.2.2 SENSITIZATION VIA OTHER ROUTES

Allergens can enter the body through the airways, the gastrointestinal tract, or the skin. Atopic children with defects in the skin barrier may become sensitized to a food allergen without prior oral contact to the allergen.¹¹ The oral ingestion of allergenic proteins is suggested to promote tolerance, whereas skin contact leads to sensitization. Filaggrin mutation, which impairs the skin barrier, is a risk factor in peanut sensitization,^{12,13} and recent evidence shows that early peanut introduction promotes oral tolerance.¹⁴

2.3 CHARACTERISTICS OF A NUT ALLERGY

2.3.1 PREVALENCE OF NUT ALLERGIES

Publications on nut allergy prevalence report sensitization to nuts, self-reported allergies, and challenge-confirmed allergies. In a Finnish study, the prevalence of parent-reported nut allergy was 3.1% in children starting elementary school.¹⁵ Of Finnish students with atopy, 23% reported symptoms from tree nuts and 17% from peanut.¹⁶

In a meta-analysis, peanut allergy prevalence in Europe was 0.4% for overall self-reported lifetime prevalence, while skin prick test positivity was 1.7%. The most prevalent in this regard was IgE positivity at 8.6%. The challenge-confirmed peanut allergy accounted for 0.2%. The prevalence estimates were higher in Western Europe compared to other regions.¹⁷

In tree nut allergies, the overall self-reported lifetime prevalence was 1.3%, skin prick test positivity 0.6% and challenge positivity 0.5%. Tree nut allergy estimates were higher in Northern Europe.¹⁷ A systematic review in 2015 reported tree nut allergy prevalence in Europe, the USA, and the UK and included almond, Brazil nut, cashew, hazelnut, macadamia, pecan, pistachio and walnut. Prevalence of challenge-confirmed tree nut allergy was less than 2%. Prevalence estimates that included tree nut allergy with oral allergy syndrome (OAS) were higher, at 8 to 11.4%, and originated mainly from Europe. Hazelnut was the most common tree nut allergy in Europe, whereas cashew and walnut were most common in the USA.¹⁸

In a study of European adult population, hazelnut was the most common food sensitization (9.3%), and it correlated with sensitization prevalence to the birch allergens Bet v 1 and Bet v 2.¹⁹ Allergy prevalence may differ by ethnic

background; for instance in Australia, Asian-born children have higher rates of peanut allergy than their Australian-born peers,²⁰ while in South Africa, black children have lower peanut allergy rates compared to mixed-race children.²¹

2.3.2 ALLERGY ONSET

Nut allergy usually begins at an early age.²² In Australian studies, the prevalence of peanut sensitization peaked at 12 months, and 90% of peanut allergy developed by six years of age.^{23, 24} In a US voluntary registry, the median age for the first peanut reaction was 14 months, and for tree nuts it was 36 months.²⁵ In contrast to early-onset allergy, late-onset nut allergy can be confused more easily with a cross-allergy to pollen.

2.3.3 SYMPTOMS

Nut allergy symptoms range from mild oral itching to anaphylaxis, which is defined as a severe, life-threatening generalized or systemic hypersensitivity reaction.²⁶ Anaphylaxis usually occurs within 2 hours of allergen exposure,²⁷ and in food allergies it is usually within 30 minutes.²⁸ The first-line rescue medication is intramuscular adrenaline.²⁹

2.3.4 RISK RATES AND PROGNOSIS

Nuts are the cause of most severe and even fatal allergic reactions associated with food, with fatalities occurring especially in adolescents and young adults.³⁰ The annual rate of accidental peanut exposure is estimated to be 12% in children with a peanut allergy,³¹ whereas data on other nuts is scarce. General food anaphylaxis incidence is 0.14 per 100 person-years, and it is highest in young children.³² The incidence of fatal food anaphylaxis is 1.81 per million person-years in food-allergic people.³³ Peanut is commonly reported as the most common trigger of food and nut reactions. In Sweden, peanut, cashew, and hazelnut were the most common nut allergies leading to emergency department visits.³⁴ In a study of anaphylaxes, peanut was one of the most prevalent elicitors at all ages, and at preschool age, hazelnut and cashew also elicited anaphylaxes.³⁵ A German study reported foods as causing 65% of severe allergic reactions in children, with peanut being the most common trigger (17%), and hazelnut was third (8%).³⁶ Reports of trigger foods in emergency settings may be biased toward easily identified nut species. A nut allergy is usually a life-long burden, though peanut allergy can be outgrown in 20% and tree nut allergy in 10% of patients.³⁷ In young children, peanut allergy resolves in up to 22%, and a small skin prick wheal and a low IgE level predict resolution.³⁸ The spontaneous resolution of peanut allergy happens mainly before six years of age, and after ten years of age, natural resolution is infrequent.³⁹

2.4 NUT ALLERGENS

Allergenic plant proteins comprise both labile and stable allergens.⁴⁰ Molecular allergology examines sensitization to specific allergenic molecules instead of the whole allergen source. In plant-derived foods, seed storage proteins are stable allergens that preserve their conformation in food processing (i.e. heating, boiling) and in gastric digestion. Sensitization to seed storage proteins is therefore associated with severe allergic symptoms. In addition to seed storage proteins, lipid transfer proteins (LTPs) are stable proteins and considered to associate with severe symptoms. LTPs can act as pan-allergens, causing cross-sensitization between many species. However, most pollen-cross-reactive allergens are usually labile and unassociated with severe symptoms, as food processing and gastric digestion destroy their structure.

Table 1. Classification of the most important allergen families in nuts. Data obtained from Molecular Allergology Users Guide: Part A Molecular Allergology: General concepts: Allergen families and databases.⁴¹

	Characteristics
Prolamins - 2S albumins - Non-specific lipid transfer proteins (nsLTPs) (PR-14)*	Resistant to heat denaturation and gastric digestion Species-specific allergens, except nsLTPs, which can act as pan-allergens
Cupins - Legumins (11S globulins) - Vicilins (7S globulins)	
Pathogenesis-related (PR) proteins - PR-10 proteins - PR-14 proteins (Non-specific lipid transfer proteins)*	PR-10 group: Sensitive to heat denaturation and gastric digestion, wide cross-reactivity between species PR-14 group: Resistant to heat denaturation and gastric digestion, can act as pan-allergens
Profilins	Sensitive to heat denaturation and gastric digestion, wide cross-reactivity between species, common pan-allergens
Oleosins	Oil body-associated proteins, clinical relevance is uncertain

* Classified as both prolamins and pathogenesis-related proteins

Table 2. Characterized allergens in nut species. 42, 43, 44, 45, 46, 47

	Seed storage proteins			Pathogenesis-related proteins		Oleosins	Profilins	Defensins
	11S globulin	7S globulin	2S albumin	PR10-protein	nsLTP			
Peanut <i>Arachis hypogaea</i>	Ara h 3	Ara h 1	Ara h 2 Ara h 6 Ara h 7	Ara h 8	Ara h 9 Ara h 16 Ara h 17	Ara h 10 Ara h 11 Ara h 14 Ara h 15	Ara h 5	Ara h 12 Ara h 13
Hazelnut <i>Corylus avellana</i>	Cor a 9	Cor a 11	Cor a 14	Cor a 1	Cor a 8	Cor a 12 Cor a 13	Cor a 2	
Almond <i>Prunus dulcis</i>	Pru du 6		Pru du 2S albumin ⁴⁴	Pru du 1 ⁴⁵	Pru du 3		Pru du 4	
Cashew <i>Anacardia occidentale</i>	Ana o 2	Ana o 1	Ana o 3					
Pistachio <i>Pistachia vera</i>	Pis v 2 Pis v 5	Pis v 3	Pis v 1					
Walnut <i>Juglans regia</i>	Jug r 4	Jug r 2	Jug r 1	Jug r 5 ⁴⁶	Jug r 3			
Pecan <i>Carya illinotensis</i>	Car i 2 Car i 4 ⁴⁷		Car i 1					
Brazil nut <i>Bertholletia excelsia</i>	Ber e 2		Ber e 1					
Macadamia <i>Macadamia integrifolia</i>								
Coconut <i>Cocos nucifera</i>	Coc n 4	Coc n 2					Coc n 5	

2.5 POLLEN-INDUCED CROSS-REACTIVITY

Allergen cross-reactivity is a phenomenon in which an IgE molecule, produced specifically against a certain allergen, binds to a similar structure on another allergen. Cross-reactivity can exist between various allergenic proteins and between allergen sources from differing species. In pollen-induced cross-reactivity, pollen is the primary sensitizer and can cause cross-reactivity to various plant-derived foods.^{48, 49} Some cross-reactive species pose a similar botanical origin. In cross-reactivity, which is caused by similar species-specific proteins, similar botanical origin is more common than in cross-reactivity caused by pan-allergens. Pan-allergens, such as lipid transfer proteins or profilins, can reside in species that are botanically distant.⁵⁰ Thus, botanical origin only partly explains allergological cross-reactivity.

2.5.1 BIRCH POLLEN SENSITIZATION

Tree pollen is the most common inhalant allergen causing cross-sensitizations to foods.⁴⁸ Finland has a very high birch pollen count⁵¹ and birch sensitization is the most prevalent pollen sensitization in Finland and across Northern Europe.⁵² Birch sensitization starts to occur after infancy and the rate increases until adulthood with young adults having the highest rates.⁵³ Of young adults in Finland, 28% are sensitized to birch pollen,⁵⁴ and sensitization is directed to the major allergen Bet v 1 in 98% cases. Bet v 2 is the second most common allergen, but it accounts only 2% of sensitization.⁵⁵ In Swedish, Austrian, and French populations, Bet v 1 accounts similarly for over 90% of sensitization.^{55, 56}

2.5.2 ALLERGENS AND ORAL ALLERGY SYNDROME

Bet v 1 is the primary sensitizing agent in birch-associated food allergies,⁵⁷ and it drives sensitizations against other PR-10 proteins.⁵⁸ Of nuts, PR-10 proteins are characterized in almond, hazelnut, peanut,⁵⁹ and walnut.⁴⁶ Birch pollen-related food allergy associates with Bet v 1 sensitization.⁵⁶ In general, sensitization to PR-10-proteins does not cause severe reactions, since labile allergens are unable to cause a primary food allergy via the gastrointestinal tract, as they are destroyed before entering the gut.⁶⁰ PR-proteins can cause oral allergy syndrome (OAS) which is a mild form of food allergy and includes itching or tingling of the mouth, lips, throat, or ears.⁶¹ In pollen season, patients may be more prone to experience OAS symptoms.^{56, 62}

2.5.3 PREVALENCE AND COMMON TRIGGER FOODS

Of birch-allergic individuals, 70% are estimated to experience symptoms from

birch-related foods.^{57, 56} Subjects are more prone to experience symptoms from foods if they have high IgE to Bet v 1⁵⁶ or if they experience symptoms from pollen.⁶³ In addition to OAS, another term, pollen food syndrome (PFS), is used to describe the phenomenon and possible symptoms of cross-sensitization to pollen and foods. In the UK population, the majority of patients report experiencing the first symptoms of PFS before the age of 20 years.⁶⁴

As the allergen protein families that may cause OAS are found widely in the plant kingdom, potential trigger foods for OAS are manifold. Reported OAS-inducing foods include apple, carrot, hazelnut, walnut, celery, soybean, pear, peach, nectarine,⁵⁶ kiwifruit,⁶⁵ peanut, almond, mungbean, tomato, potato, plum, apricot, cherry,^{48, 64} and jackfruit.⁶⁶ Individual fruits and vegetables differ in their symptom potency as the allergen content depends on the cultivar and ripeness.⁶⁷

Of the nuts, hazelnut especially causes symptoms in birch-allergic individuals.^{56, 64, 68} In the UK, 25-30% of PFS patients reported hazelnut as a trigger food for pollen-related food allergy.⁶⁴ Birch-allergic Austrians reported apple (80%) and hazelnut (59%) as the most common triggers of food allergy.⁵⁶ Moreover, in a study of hazelnut-sensitized children, half were not clinically allergic to hazelnut despite their sensitization, and children with OAS as their only symptom were all sensitized to birch pollen.⁶⁹ Similarly, Belgian patients with OAS as their only symptom were sensitized to the Bet v 1-homologue Cor a 1 in hazelnut. Adults mainly had OAS as their only symptom whereas systemic reactions were more common in children.⁷⁰ Sensitization to stable vicilin Cor a 11 occurred in patients with severe reactions to hazelnut, but not in OAS patients. Again, severe reactions were more common in children.⁷¹ The findings of these smaller studies were verified in a Europe-wide study, in which birch-pollen-associated hazelnut sensitization predominated in adulthood, and sensitization to stable allergens was more common in children.⁷²

In line with hazelnut, peanut sensitization is affected by birch pollen sensitization. In a Swedish study, children were less likely to report symptoms if they were simultaneously birch-sensitized, compared to children that were only peanut-sensitized.⁷³ Peanut was the eleventh most common trigger of birch-associated food allergy, with 24% reporting symptoms in the Austrian study. Almond was reported by 32% and walnut by 41% of the responders.⁵⁶ In the UK, cashew/pistachio caused symptoms in 10-15% and Brazil nut in 20-25% of PFS patients. In general, nuts were the third most common trigger of PFS.⁶⁴ The prevalence of macadamia or coconut as triggers of pollen-related symptoms was not reported.^{56, 64}

2.5.4 GRASS AND WEED POLLEN SENSITIZATIONS

In addition to birch, grass pollen sensitization is common with a 17% prevalence rate in developed countries.⁷⁴ Phl p 1, an allergen belonging to a

protein family of expansins, is the major allergen.⁷⁵ Grass pollen allergy is not associated with food allergen cross-sensitizations as much as birch.^{76, 63} In addition to the PR-10 proteins, profilins are another family that can cause cross-sensitization between pollens and plant-derived foods, but it seems that profilin sensitization rarely leads to clinical symptoms.^{48, 76, 77} Furthermore, birch pollen-sensitized patients with profilin Bet v 2 sensitization are not as prone as Bet v 1-sensitized subjects to experiencing symptoms from foods.⁵⁶ Weed pollinosis may induce pollen food syndrome, though not as frequently as birch pollinosis.^{48, 78} Mugwort (*Artemisia vulgaris*) causes mugwort-celery-spice- and mugwort-mustard syndromes, and ragweed (*Ambrosia artemisiifolia*) has cross-reactivity with melon and banana. Several allergens, including labile and stable proteins, can be responsible of these cross-reactivities.⁷⁸

2.5.5 OTHER CROSS-REACTIVE DETERMINANTS

Lipid transfer proteins (LTPs) are pan-allergens, and in contrast to PR-10 proteins and profilins, they are stable and can cause symptoms also in processed foods. LTP sensitization is considered to originate from primary sensitization to peach LTP Pru p 3, but primary sensitization to pollen allergens is also possible.⁷⁹ Sensitization to LTPs in peanut⁸⁰ and hazelnut⁸¹ can cause severe allergy, but in Northern and Central Europe, LTP sensitization is infrequent.⁸² In Southern Europe, LTP sensitization is more common, possibly due to differences in pollen exposure or culinary habits.^{79, 83, 72} In addition to peptides of proteins, carbohydrate structures on proteins can bind IgE and cause cross-sensitization. The IgE binding to carbohydrates of plant allergens is considered fairly harmless.^{84, 85}

2.6 POLLEN-INDEPENDENT CROSS-REACTIVITY BETWEEN NUT SPECIES

Cross-sensitization and cross-allergy occur between nut species as well as between nuts and other plant-derived foods, without any interference from pollen sensitization. The underlying allergens are species-specific and can include seed storage proteins and other stable allergens. Hazelnut, cashew, peanut, and walnut share similar IgE binding epitopes in their vicilin allergens, which may explain part of the cross-reactivity between these species.⁸⁶ A Dutch study showed that in a birch-endemic region, sensitizations based on seed storage proteins in hazelnut and peanut are mostly independent.⁸⁷ Despite the independence of pollen sensitization, concurrent pollen sensitization may still be present, especially in regions where pollen sensitization is very prevalent,⁸⁸ and without molecular allergology, cross- or co-sensitization cannot be separated.

2.7 NUT ALLERGY DIAGNOSIS

The accurate diagnosis of a nut allergy is important, in order to keep severely allergic patients safe by giving the right patient information and prescribing emergency medication. Unnecessary avoidance diets are burdensome and can lower the quality of life.^{89,90} Cross-reactivity with pollen and plant-derived foods complicates diagnostics.

2.7.1 PATIENT HISTORY

Immediate symptoms, starting in minutes through 2 hours after exposure to the suspected allergen, are indicative of an IgE-mediated reaction. Anaphylaxis or other immediate multisystem symptoms raise the suspicion of an IgE-mediated allergy. If the patient has eaten the suspected food previously without symptoms, an allergy is less likely, albeit quantity and food processing may affect reactivity.⁹¹ Augmenting factors, such as physical exercise, illness, medications, and menstruation, may lower the reaction threshold or worsen allergy symptoms.⁹² As nuts may already cause symptoms in small amounts,⁹³ hidden allergens are possible triggers of symptoms, and small children especially may have no known exposure to the suspected food.

2.7.2 SKIN PRICK TESTS IN THE DIAGNOSIS OF NUT ALLERGY

The purpose of skin prick testing is to examine sensitization to an allergen and thereby interpret the clinical allergy. The method should always include positive and negative controls.⁹⁴ Test extracts commonly include the whole allergen source, i.e. a fruit, nut, pollen, or animal dander, but allergen-component-based testing is also possible.⁹⁵ Testing with raw products, in the case of fruits, vegetables, or nuts, is considered more sensitive than extract-based testing, as labile allergens are preserved in the raw products. However, the allergenic content of natural products can vary according to ripeness or the cultivar.⁶⁷ As a skin prick test (SPT) is an in vivo test, it is regarded also as a “mini-challenge”, and it poses a possible risk of systemic allergic reaction. The highest risk for severe reactions is considered with nuts.⁹⁶ SPT wheal size is a continuum, and several cut-offs for clinically significant sensitization in different allergens have been proposed. With bigger wheal sizes, a clinical allergy is more probable.⁹⁷ Meta-analysis on skin prick test in peanut allergy diagnosis showed 95% sensitivity, but only 61% specificity in pooled analysis when a cut-off was set to 3mm.⁹⁸ Negative (<3 mm) skin tests have a good negative predictive value for clinical allergy.^{99, 100, 101, 102, 103} In peanut allergy, the cut-off for a 95% positive predictive value (PPV) would be at least 7 to 8

mm, and for a 100% PPV this would rise to 15 mm.¹⁰³ Wheals between 3 to 7 mm are somewhat of a gray area, in that an allergy or tolerance is difficult to determine.¹⁰⁴ In general, in young children, the cut-off for clinical allergy can be lower than in older children or adults.^{103, 99, 104, 105} A study on peanut and several tree nuts in children and adolescents reported that under 3 mm wheals showed over 90% negative predictive value in most species. A high positive predictive value was reached in most species at 6 to 8 mm wheals.⁹⁹

2.7.3 SERUM IGE TESTING IN THE DIAGNOSIS OF NUT ALLERGIES

A common singleplex test (one assay per sample) for detecting specific IgE in serum is based on a sandwich immunoassay, in which the allergen is immobilized on a solid phase. IgE in the patient serum sample binds the allergen molecule. After washing away unbound non-specific IgE, fluorescence-labeled anti-IgE antibodies bind to the allergen-IgE-complexes and the measured fluorescence corresponds to the quantity of specific IgE in the sample.⁴² As in the skin prick test, both whole-nut extracts as well as specific allergen components, from either natural or recombinant origins, can be used. In natural allergens, carbohydrate determinants may cause unspecific IgE binding. As in the skin prick test, IgE levels are a continuum and a specific cut-off for clinical allergy is difficult to determine. Cut-offs between 10 and 19 kU/L are proposed to predict at least 95% probability in peanut, hazelnut, and walnut allergies.^{104, 106, 105, 107}

2.7.3.1 Component-specific IgE and clinically significant levels

The use of molecular allergology has improved allergy diagnostics markedly. The measurement of specific IgE to seed storage protein components, especially 2S albumins, has increased both the sensitivity and the specificity of nut allergy diagnosis to over 90 percent. The relevance of the allergen components of several nut species has been studied in clinical settings through challenge tests.

Peanut

Allergen components have been most extensively studied in peanut allergy. The stable seed storage proteins Ara h 1, 2, 3, and 6 are usually responsible for severe reactions, and sensitization simultaneously to Ara h 1 (vicilin) and Ara h 3 (glycinin), in addition to the 2S albumins, indicates more severe reactions.^{95, 108} The 2S albumins Ara h 2 and Ara h 6 are the best predictors of clinical peanut allergy.^{109, 110, 111}

The diagnostic accuracy of Ara h 2 has been studied in different patient populations in Europe, Australia, US, Canada, Asia, and South Africa.^{111, 112, 113}

In a review including studies running up until 2013, the sensitivity of Ara h 2 ranged between 60 and 100%, and specificity between 60 and 96%.¹¹¹ In Finnish children and adolescents, Ara h 2 with 1.8 kU/L showed 80% sensitivity and 95% specificity.¹⁰⁹ In the German pediatric population, a cut-off of 0.35 kU/L for Ara h 2 had 86% sensitivity and 86% specificity.¹¹⁴

Ara h 6 has not been studied as widely as Ara h 2. They both belong to 2S albumins and have high sequence homology¹¹⁵ as well as similarities in surface structures.¹¹⁶ In the Finnish study, Ara h 6 with a cut-off of 0.8 ISU-E showed 95% sensitivity and specificity.¹⁰⁹ Ara h 6 sensitization can also occur as monosensitization in rare cases, but equally to Ara h 2, it has the potential to induce severe reactions.^{117, 118}

Monosensitization to the cross-reactive PR-10 protein and Bet v 1-homologue Ara h 8 indicates tolerance.¹¹⁹ However, PR-10 proteins are suggested to cause symptoms when large amounts of the allergen are ingested into an empty stomach and/or physical exercise is combined with allergen exposure.⁴¹ One case report has been published, in which large amount of peanut ingested into an empty stomach caused anaphylaxis in an Ara h 8-monosensitized patient.¹²⁰ The lipid transfer protein Ara h 9 is an important peanut allergen in the Mediterranean,⁸⁰ but similar to other regions, Ara h 2 and Ara h 6 still exhibit the best discriminative ability in Mediterranean child populations.^{117, 121} As LTP allergens reside near the peel of a fruit or nut, peeled products may lose their allergenicity in patients that are monosensitized to Ara h 9.¹²²

Hazelnut

Hazelnut 2S albumin Cora 14 is responsible for clinical hazelnut allergy similarly to peanut 2S albumins. In a Europe-wide study on self-reported hazelnut allergy, fewer than 10% of subjects were sensitized to seed storage proteins. Sensitization to the Bet v 1-homologue Cor a 1 dominated in most regions, and LTP Cor a 8 sensitization was again most prevalent in the Mediterranean. Sensitization to oleosin allergens occurred in all regions, though its clinical relevance was stated as uncertain.⁷²

Seed storage proteins Cor a 9 (legumin) and Cor a 14 (2S albumin) were the most useful allergens in Dutch children and adults. A cut-off 0.35 kU/L for Cor a 14 showed 70% sensitivity and 76% specificity.¹²³ In the German child population, Cor a 14 had the best discriminative ability with a cut-off of 0.35 kU/L yielding 85% sensitivity and 81% specificity.¹¹⁴ In Mediterranean children, Cor a 14 was the best for discriminating hazelnut allergy, with an optimal cut-off of 0.63 kU/L, sensitivity 81.8%, and specificity 100%.¹⁰⁵ In Danish children, a cut-off 0.72 kU/L for Cor a 14 specific-IgE diagnosed 87% correctly.¹²⁴

Cashew and pistachio

In cashew allergy, 2S albumin Ana o 3 was able to discriminate effectively between cashew as well as pistachio allergy in Greek children. A cut-off of 0.16 kU/L yielded 98% sensitivity and 94% specificity.¹²⁵ In Dutch children, seed storage proteins Ana o 1, 2, and 3 accurately discriminated tolerant from allergic, but the superiority of any of the three seed storage proteins was not reported.¹²⁶ In German children, Ana o 3 was a good predictor of a clinically relevant allergy, and with a cut-off of 0.3 kU/L, sensitivity was 93% and specificity 90%.¹²⁷

In discriminating pistachio allergy in Greek children, cashew Ana o 3 showed 97% sensitivity and 94% specificity when the cut-off was set to 0.16 kU/L.¹²⁵ Pistachio allergens have been studied in vitro. 2S albumin Pis v 1 was the leading IgE reactive protein in an in vitro study.¹²⁸ Vicilin, Pis v 3, cross-reacts with the homologous Ana o 1 from cashew. IgE reactivity to Pis v 3 was found in patients with allergy to both pistachio and cashew, or those who were cashew-allergic but had never eaten pistachio.¹²⁹

Walnut and pecan

Specific IgE to Jug r 1, 2S albumin in walnut, has the highest discriminative ability in walnut allergy. LTP Jug r 3 was not a relevant allergen in Dutch and British studies.^{130, 131} Sensitization to vicilin Jug r 2 from ImmunoCAP, a native allergen, was associated with sensitization to cross-reactive carbohydrate determinants and did not indicate a clinical allergy.¹³² In addition, Jug r 2 in the ISAC microarray exhibited low discriminative ability.¹³⁰ Similar to peanut and hazelnut, Jug r 1 is the most prevalent sensitization in Northern and Central Europe and Northern America, whereas LTP Jug r 3 predominates in Southern Europe.¹³³

Pecan 2S albumin Car i 1 was characterized as a major allergen in an in vitro study, in which 79% of patients' sera bound to Car i 1.¹³⁴ A vicilin Car i 2 and a legumin Car i 4 also bind IgE in pecan allergic patients' sera, but not as commonly as 2S albumin Car i 1.^{47, 135}

Other nuts

Studies on almond allergy are very limited. Publications on chemical characteristics of almond 2S albumin are somewhat controversial and its clinical characteristics have not been studied.^{136, 44, 45} Pru du 6 (11S globulin) is a major almond allergen with up to 50% of patients' sera binding to it in an in vitro study.¹³⁷

Brazil nut 2S albumin has been assessed in a clinical study. Ber e 1 yielded 75% sensitivity and 94% specificity with a cut-off of 0.25 kU/L in a study of 36 patients.¹³⁸

Publications on the allergen-component diagnostics of coconut and macadamia allergies are limited. Currently, a 7S globulin Coc n 2¹³⁹ and an 11S globulin Coc n 4 are described as coconut allergens and responsible for allergic reactions.^{140, 141} In macadamia, 12 kDa, 17.4 kDa, and 45 kDa proteins have been described as potential allergens.^{142, 143}

2.7.4 RATIOS OF SPECIFIC IGE TO TOTAL IGE AND SPECIFIC IGG4 TO IGE

Previously, the ratio of specific IgE to total IgE has been suggested as being more accurate in the diagnostics of peanut allergy than specific peanut IgE alone.¹⁴⁴ However, the ratios of component-specific IgE to total IgE do not improve peanut, hazelnut, and cashew allergy diagnostics.^{145, 127} Testing of IgG4 alone is not recommended for food allergy diagnostics.¹⁴⁶ However, as IgG4 antibodies may indicate tolerance, the level of specific IgG4 compared to specific IgE might offer greater accuracy for food allergy diagnostics. Nonetheless, patients with high IgE may produce the highest IgG4 levels and therefore, the IgG4-to-IgE ratio is inferior to IgE alone.^{147, 148} In birch-pollen-allergic patients, IgG4-to-IgE ratios to apple and hazelnut PR-10 allergens are lower in those patients who experience symptoms from these foods, but as the ratios are highly variable, they are not suitable for diagnostics of birch pollen-related food allergy.⁵⁶

2.7.5 MICROARRAY

In addition to the singleplex assay, a multiplex microarray can be used to measure IgE simultaneously for several allergens. The array includes immobilized allergens that bind IgE from one sample. A commercial Immuno Solid-phase Allergen Chip (ISAC) includes 112 predefined allergens that originate from 51 allergen sources. Both recombinant and natural allergens are included. The method of IgE detection is based on immunofluorescence and is semiquantitative.¹⁴⁹ The detection limit is 0.3 ISU-E (ISAC standardized units for specific IgE), which is usually also considered as a cut-off for positive. The array comprises foods of plant and animal origins, pollens from trees and grasses, animal dander, latex, mite, mold and insect venoms. Of the allergenic protein families present in nuts, it includes 10 PR-10 allergens, 14 seed storage proteins, four profilins, and nine LTPs. Of nuts and seeds, it includes six allergens from peanut, three from hazelnut, one from sesame seed, one from cashew, three from walnut, and one from Brazil nut. (Table 3). An important use for an allergen microarray is to screen IgE for a large number of possible trigger allergens in cases of unspecific anaphylaxis.¹⁵⁰ The array is more expensive than singleplex tests, but when larger numbers of singleplex tests are needed, the array is cost-effective.¹⁵¹ A disadvantage of a predefined panel is that it may provide results that are not of interest in a specific patient

and only lead to unnecessary and costly further examinations which distress the patient and family. ^{151, 152}

The microarray has markedly lower amounts of allergen than singleplex tests, which may lead to competition in antibody binding. IgG4 antibodies can act as blocking antibodies in the IgE microarray and result in falsely low results of IgE, if the amount of allergen binds primarily to excess IgG4. ¹⁵³

In immunotherapy, this competitive binding may be of use when increasing IgG4 leads to lower IgE results. This approach could even be beneficial in monitoring the immunotherapy response. ^{154, 155}

Table 3. Allergens in the Immuno Solid-phase Allergen Chip (ISAC)
(ImmunoCAP ISAC®, Thermo Fisher Scientific, Uppsala, Sweden)

Plant foods:	Grass pollen:
Kiwi (Act d 1, 2, 5, 8)	Bermuda grass (Cyn d 1)
Celery (Api g 1)	Timothy (Phl p 1, 2, 4, 5, 6, 7, 11, 12)
Apple (Mal d 1)	
Peach (Pru p 1, 3)	Weed and flower pollen:
Cashew (Ana o 2)	Ambrosia (Amb a 1)
Brazil nut (Ber e 1)	Mugwort (Art v 1, 3)
Hazelnut (Cor a 1.0401, 8, 9)	Chenopodium album (Che a 1)
Walnut (Jug r 1, 2, 3)	Mercurialis annua (Mer a 1)
Sesame (Ses i 1)	Parietaria judaica (Par j 2)
Peanut (Ara h 1, 2, 3, 6, 8, 9)	Plantago lanceolata (Pla l 1)
Soy (Gly m 4, 5, 6)	Salsola kernerii (Sal k 1)
Wheat (Tri a 14, 19.0101, aA_TI)	
Buckwheat (Fag e 2)	
Animal foods:	Mites:
Milk (Bos d 4, 5, 8, Bos d lactoferrin)	Dermatophagoides (Der p 1, 2, 10, Der f 1, 2)
Bovine serum albumin (Bos d 6)	Blomia tropicalis (Blo t 5)
Ovomucoid (Gal d 1)	Lepidoglyphus destructor (Lep d 2)
Ovalbumin (Gal d 2)	
Conalbumin (Gal d 3)	Parasites:
Egg yolk (Gal d 5)	Anisakis simplex (Ani s 1, 3)
Cod (Gad c 1)	
Shrimp (Pen m 1, 2, 4)	
Mold:	Cockroaches and insects:
Alternaria (Alt a 1, 6)	Cockroach (Bla g 1, 2, 5, 7)
Aspergillus (Asp f 1, 3, 6)	
Cladosporium (Cla h 8)	Animals:
Tree pollen:	Dog (Can f 1, 2, 3, 5)
Alder (Aln g 1)	Cat (Fel d 1, 2, 4)
Birch (Bet v 1, 2, 4)	Mouse (Mus m 1)
Hazel tree (Cor a 1.0101)	Horse (Equ c 1, 3)
Cypress (Cup a 1)	
Japanese cedar (Cry j 1)	Insect venoms:
Olive tree (Ole e 1, 7, 9)	Bee venom (Api m 1, 4)
Plane tree (Pla a 1, 2, 3)	Vasp venom (Pol d 5, Ves v 5)
	Other:
Latex (Hev b 1, 3, 5, 6.01, 8)	Bromelain-derived cross-reactive carbohydrate chain

Allergen families on the ISAC platform that are of special interest in nut and birch pollen sensitizations:

Seed storage proteins:

- 2S albumins: Brazil nut Ber e 1, Peanut Ara h 2 and Ara h 6, Walnut Jug r 1, Sesame Ses i 1
- 11S globulins: Cashew Ana o 2, Hazelnut Cor a 9, Peanut Ara h 3
- 7S globulins: Walnut Jug r 2, Peanut Ara h 1

PR-10 proteins:

Peanut Ara h 8, Hazelnut Cor a 1.0401, Hazel tree pollen Cor a 1.0101, Birch tree pollen Bet v 1

Lipid transfer proteins:

Hazelnut Cor a 8, Walnut Jug r 3, Peanut Ara h 9

2.7.6 FOOD CHALLENGE

The double-blind placebo-controlled food challenge is the gold standard of food allergy diagnostics.² Challenge protocols vary according to the starting dose, the time interval between doses, the number of doses, and the cumulative dose. The American and European Practical Allergy Report (PRACTALL) recommends conducting the double-blind placebo-controlled challenge over two days.¹⁵⁶ In addition to the patient, the caregiver, the nurse, and the treating physician should be blinded. After a blinded active challenge without symptoms, the challenge can be continued openly with a higher dose. Antihistamines and other medications with antihistaminic properties should be avoided before the challenge.¹⁵⁶

2.7.6.1 Dosing

The cumulative allergen dose during the challenge should be at least 2g of food protein. The PRACTALL recommends 3-10-30-100-300-1000-3000 mg doses of protein and at least 20 min intervals between these doses.¹⁵⁶ Longer intervals have been studied in peanut challenges, and up to 2-hour time intervals may reflect better the threshold for symptoms.¹⁵⁷

2.7.6.2 Safety considerations

The food challenge poses a risk of severe allergic reaction, and so emergency

care should be available. Risk factors for anaphylaxis in children's challenges are older age and peanut as the challenge food.¹⁵⁸ According to PRACTALL, a challenge should not be conducted if the patient is experiencing unstable or exacerbated atopic eczema, asthma, urticaria, or allergic rhinitis. Furthermore, if the patient has a health condition that poses special risks when treating anaphylaxis, i.e. unstable angina pectoris, cardiac disease or dysrhythmias, severe chronic lung disease, or pregnancy, the challenge should not be performed.¹⁵⁶

2.7.6.3 Interpretation of the challenge result

The challenge should be stopped and judged as positive following the emergence of objective symptoms. In the case of subjective symptoms, the options are to discontinue the challenge, wait longer until the next dose, or repeat the previous dose. If the symptoms worsen, persist for at least 45 min, or occur repeatedly after three allergen doses, the challenge should be judged as positive. Undertaking several challenges with both placebo and active preparations is time-consuming, but it might be needed if the result remains inconclusive.¹⁵⁶ Subjective symptoms associate with the most variability in challenge interpretation.¹⁵⁹ Reactions to a placebo are possible, but in a study of over 700 challenges, only 2.8% of placebo challenges were interpreted as positive. Most reactions involved the worsening of atopic eczema, and these tended to occur in young children.¹⁶⁰

2.7.6.4 Challenge preparation

Food matrix development requires the sufficient masking of allergenic food in relation to taste, smell, and appearance.¹⁶¹ The amount of allergenic food should be maximized in the final preparation, in order not to result in a very high cumulative dose. Furthermore, patients' other allergies may limit the possible masking ingredients. The matrix must not affect the absorption of the allergen. High fat content may slow absorption, mask early symptoms, and eventually lead to more severe symptoms.¹⁶² Finally, processing may alter the allergenicity of proteins. Roasting enhances and boiling reduces the allergenicity of peanut seed storage proteins.^{163, 164}

2.7.6.5 Reaction threshold and severity

One purpose of a food challenge is to examine the eliciting dose for symptoms in an individual patient. However, augmenting factors may alter the threshold, and it is generally not reproducible.¹⁶⁵ Eliciting dose in 5% of Dutch children was 1.6 mg protein for peanut, 0.29 mg for hazelnut, and 7.4 mg for cashew.¹⁶⁶ Assessment of symptoms in a quantitative scale is especially useful in research settings. In addition to the type of symptoms and required treatment, the severity of the reaction can be scored according to the eliciting dose.¹⁶⁷

2.7.6.6 Life quality effects and reintroduction of the food

In a study of patients undergoing double-blind placebo-controlled challenges, the health-related quality of life improved both in the challenge positives as well as -negatives, though the improvement was greater in the challenge negatives.¹⁶⁸ After a negative challenge, patients should be encouraged to reintroduce the food into their diet. One study reported 28% of children failing the introduction after a negative challenge. The reasons associated with failure were experience of symptoms, aversion, fear, habit, other allergies, the patient considering the challenge positive, and that the family was allergic to many things in general.¹⁶⁹

2.8 IMMUNOTHERAPY FOR PEANUT ALLERGY

The purpose of immunotherapy is to elevate the threshold dose for symptoms and prevent severe reactions in accidental exposures. Desensitization is a temporary state of unresponsiveness to an allergen and requires the continuous consumption of the allergen. Tolerance is a permanent state of desensitization in which even a long off-treatment period does not lead to symptoms in an allergen exposure.^{41, 170} The first published study on immunotherapy for food allergy dates back to 1908, when a case report of egg OIT was published.¹⁷¹ Since then, studies on immunotherapies to milk, egg, wheat, peanut, hazelnut, cashew, walnut, soy, and apple have been published. Reports on immunotherapies for nut allergies other than peanut are scarce.^{172, 173} A recent meta-analysis stated that food allergy immunotherapy may be effective in raising the threshold of reactivity in children with IgE-mediated food allergy, both during and post-discontinuation of the treatment. Nonetheless, the treatment was stated to be associated with an increased risk of adverse reactions.¹⁷⁴

2.8.1 STUDIES ON PEANUT IMMUNOTHERAPY

According to guidelines set out by European⁹ and American allergy and clinical immunology associations,⁹¹ and the national Finnish working group,³ immunotherapy for any food allergy is currently recommended for research settings only. Most current studies on peanut immunotherapy report oral administration of the allergen.

In 1992 subcutaneous immunotherapy for peanut allergy was reported. The study was terminated early as a formulation error in the pharmacy caused lethal anaphylaxis in a placebo group patient. Otherwise, the authors considered the treatment effective and the rate of systemic reactions (13.3%) acceptable.¹⁷⁵ In 1997, immunotherapy with injections of aqueous peanut extract showed efficacy, but a high rate of systemic reactions led to the conclusion that clinical application of the treatment requires modified peanut extracts.¹⁷⁶ Sublingual administration of peanut immunotherapy has been reported in several studies.^{177, 178, 179, 180, 181} A US study compared oral and sublingual administration and concluded that the safety profile is better in sublingual administration, but efficacy is worse.¹⁷⁹ Epicutaneous peanut immunotherapy is currently being studied^{182, 183} and seems safe, but only modest efficacy has been reported.¹⁸² One study reported rectal administration of recombinant peanut allergens, but it concluded that the treatment resulted in frequent adverse events.¹⁸⁴ Published studies on oral administration of peanut immunotherapy are heterogeneous regarding protocols and primary outcomes. (Table 4) Some studies have focused mainly on efficacy^{185, 186} and some on safety.^{187, 188} In addition,

immunological changes^{189, 190, 191}, protocols¹⁹², comparison of administration routes¹⁷⁹, and adjuvant therapies^{193, 194} have been studied.

2.8.2 THE INCLUSION OF PATIENTS

Some studies have confirmed the diagnosis of peanut allergy with either DBPCFC or OFC, before enrolling patients on the treatment, while other studies have included patients based on sensitization tests and patient history. (Table 4)

Patients with severe anaphylaxis (hypotension, collapse, admission to intensive care) have been excluded in several studies, and uncontrolled asthma, major chronic illness, and a lack of compliance are other major exclusion criteria. In some studies, a low threshold dose (e.g. < 100 mg peanut protein) at the baseline challenge has been an additional inclusion criterion.^{193, 195}

Patients with moderate-to-severe symptoms benefit from the therapy the most, because their risk of a severe allergic reaction is the highest. Most studies include patients 1 to 19 years of age, with the youngest patients being 9- to 36-month-olds.¹⁹⁶ (Table 4)

2.8.3 STARTING AND TARGET DOSES, BUILD-UP SCHEDULE

The starting dose in most published studies has been 0.1 mg to 5 mg of peanut protein.^{186, 179, 197, 185} The starting dose can be the same in all patients or it can be selected individually based on the baseline challenge threshold dose.¹⁹²

Dose increase usually takes place every one to two weeks, but it is postponed in case of febrile illness or allergic symptoms.¹⁹⁸ The optimal target dose for tolerance induction is unknown. In published study protocols, the target dose has been 800 to 3000 mg of peanut protein. However, lower maintenance doses (300 mg) may be equally effective in inducing immunological changes.¹⁹⁶

2.8.4 THE EFFICACY OF PEANUT ORAL IMMUNOTHERAPY

The efficacy of treatment can be assessed through several outcomes. One measure of success is the ability to increase the daily dose until the target maintenance dose is met. Peanut challenges can be conducted after the build-up phase with a higher protein dose than the daily maintenance dose, in order to assess desensitization to a larger amount of the allergen. Sustained unresponsiveness is a surrogate marker for tolerance development. It is the ability to tolerate a certain dose of peanut protein after an off-treatment period. Sustained unresponsiveness is suggested to mimic true tolerance. In published studies, the length of the off-treatment period has been few weeks or few months.^{199, 179, 196, 194}

Maintenance dose can be achieved by 74 to 97% of patients in peanut oral immunotherapy.^{199, 186, 191, 194-197} Multiple factors affect the success rate,

including target dose for maintenance, protocol compliance, and baseline IgE levels. The post build-up challenge is passed by 52 to 81% of patients.^{194-197, 199} The largest proportion of patients (81%) passed the end-challenge in the study, which included the youngest children.¹⁹⁶ The treatment failures have been described to have higher baseline peanut IgE levels compared to the treatment successes.^{196, 162}

2.8.5 IMMUNOTHERAPY PREPARATION AND THE ADMINISTRATION ROUTE

Most studies have used peanut flour as an OIT preparation.²⁰⁰ In larger doses, flour can be replaced by whole peanuts. The matrix for OIT preparation is important, because taste aversion is an important issue in oral immunotherapy to foods.²⁰¹ As food-allergic patients are commonly allergic to multiple foods, other allergies may affect matrix design. As in the food challenge, high fat content slows allergen absorption, which has to be taken into account in the safety profile of the treatment and in patient advice.¹⁶² The processing of immunotherapy preparation affects allergenicity, and enhanced immunogenicity and reduced allergenicity are favorable characteristics for immunotherapy preparation. Hypoallergenic preparations, such as boiled peanut or peptides of allergenic proteins, might be safer due to their weaker allergenicity,^{164,202} but their efficacy in inducing tolerance may be lower. Processing may also enhance allergenicity, as roasting enhances the allergenicity of peanut seed storage proteins,¹⁶³ and roasting and lipid binding may even stabilize labile PR-10 proteins.²⁰³ The route of administration affects the preservation of allergens and the efficacy of immunotherapy. Labile allergens may be destroyed in oral administration. In sublingual immunotherapy allergens are held in the oral cavity for 1 to 2 minutes before swallowing, and as allergens are absorbed already in the oral cavity, peptides of labile allergens can also encounter the local immune system.²⁰⁴

2.8.6 QUALITY OF LIFE AND MOTIVATION

Food allergen immunotherapy is a laborious treatment for both the patient and the whole family, and so quality of life has to be considered. Patients' quality of life has been reported to improve in peanut OIT.^{186, 205} The caregiver health-related quality of life was studied in a multi-allergen OIT, and it improved.^{206,207} Life quality is associated with the safety of the treatment and it is important to examine it in patients that discontinue the treatment due to any reason.²⁰⁸ Longer follow-up periods are necessary to assess the life quality effects of peanut OIT.²⁰⁹

2.8.7 ADVERSE EVENTS AND SAFETY

The majority of patients in peanut OIT experience adverse events.¹⁸⁸ Patients

often experience mild reactions, but also severe reactions, including anaphylaxis and near-fatal reactions occur. OIT may result in desensitization and protection against accidental reactions, but permanent tolerance is not usually achieved despite long-term and laborious treatment.²¹⁰ Adverse events occur more commonly in the build-up rather than in the maintenance phase.^{210, 188} Allergic rhinitis and large skin prick wheal for peanut were risk factors for adverse events in a pooled retrospective study of 104 patients. Asthma was a risk factor in the maintenance phase.¹⁸⁸ Common cofactors for adverse events are exercise, fatigue, menstruation, the use of non-steroidal anti-inflammatory drugs, the irregular intake of the allergen, and illness.¹⁹⁸ Continuous consumption of the allergenic food may induce eosinophilic esophagitis.²¹¹ A meta-analysis reported a 2.7% risk of eosinophilic esophagitis after OIT, and the disease often resolved after discontinuing OIT.²¹²

2.8.8 MAINTENANCE PHASE

The treatment is lifelong, and if it is discontinued, the achieved immunological response is usually lost.²¹³ Compliance issues should be considered carefully during lifelong treatment, and as the everyday dosing of the allergen for several years is burdensome, it may be feasible to rarefy dosing. Twice per week dosing may be equally effective in maintaining unresponsiveness.²¹⁴ Neglecting regular dosing may lead to unexpected severe reactions.²¹⁰

2.8.9 OTHER ALLERGIES AND CROSS-PROTECTION

As many peanut-allergic patients are concurrently allergic to other nuts and/or other food allergens, simultaneous immunotherapy for multiple allergens would be beneficial. However, reports on immunotherapy simultaneously for multiple foods are scarce.^{215, 173} Peanut OIT does not affect tree nut or sesame IgE levels, thus it seems that the treatment is species specific and does not offer cross-protection.²¹⁶ In a study of birch-pollen immunotherapy, concurrent hazelnut allergy showed no clinical improvement, albeit favorable immune modulation took place.²¹⁷ The risk of developing new sensitizations during immunotherapy has been studied in house dust mite allergy, and no neosensitization emerged.²¹⁸

Table 4. Published studies on oral immunotherapy to peanut. SLIT Sublingual immunotherapy; DBPCFC double-blind placebo-controlled food challenge; OFC oral food challenge; SU sustained unresponsiveness; RCT Randomized controlled trial

Study	Age	Patients	Study design	Primary diagnosis	Outcome	Maintenance dose of protein	Notifications
Anagnostou 2014 ¹⁸⁶	7-16y	49 active, 50 control	Randomized, cross-over trial	DBPCFC	24/39 (62%) passed the end-challenge	800 mg	
Clark 2009 ¹⁸⁵	9-13y	4	Open	OFC	4/4 tolerated ≥ 10 peanuts	800 mg	Pilot study for Anagnostou 2011 and 2014
Anagnostou 2011 ²¹⁹	4-18y	22	Open	OFC	19/22 (86%) achieved full maintenance dose	800 mg	
Hofmann 2009 ²²⁰	1-9y	28	Open	SPT, IgE, history	20/28 (71%) completed all 3 study phases	300 mg	Focus on safety
Blumchen 2010 ²²¹	3-14y	23	Open	DBPCFC	14/23 (61%) reached maintenance dose	500 mg	
Varshney 2011 ¹⁹⁷	1-16y	28 (19+8 placebo)	RCT	SPT, IgE, history	16/19 (84%) reached maintenance dose	4000 mg	
Vickery 2014 ¹⁹⁹	1-16y	39	Open	SPT, IgE, history	24/39 (62%) completed the protocol, 50% 1-month-SU	4000 mg	1-month-SU in 50% of patients
Jones 2009 ¹⁸⁹	1-9y	39	Open	SPT, IgE, history	29/39 (74%) reached maintenance dose	300 mg, later 1800 mg	Pilot study for Vickery 2014

Study	Age	Patients	Study design	Primary diagnosis	Outcome	Maintenance dose of protein	Notifications
Nozawa 2014¹⁹¹	5-14y	18	Rush OIT	DBPCFC	16/18 (89%) reached maintenance dose	3.5-7g peanut (875-1750 mg protein)	Rush immunotherapy, median 11 days
Narisety 2015¹⁷⁹	7-13y	21	SLIT vs OIT randomized, placebo-controlled	OFC	7/10 SLIT and 7/11 OIT achieved a 10-fold increase in OFC threshold	2000 mg	OIT had larger increase in the OFC threshold
Syed 2014¹⁹⁰	5-45y (median 10y)	43 (23 OIT+20 avoidance)	OIT vs age-matched controls on avoidance	DBPCFC	20/23 (87%) achieved maintenance dose and passed end-challenge	4000 mg	Focus on immunological changes
Bird 2015¹⁹²	4-16y	11	Open	DBPCFC	9/11 (82%) reached maintenance dose	2000 mg	Individual starting doses
Schneider 2013¹⁹⁵	7-15y	13	Omalizumab	DBPCFC (cumulative threshold ≤100 mg)	12/13 (92%) reached maintenance dose	4000 mg	Pilot study with omalizumab
Tang 2015¹⁹⁴	1-10y	62	OIT+probiotic vs probiotic only Randomized, blinded	OFC	SU was achieved by 23/31 74% in active group 26/31 84% passed 4g end-challenge in active group	2000 mg	SU was 2 to 5 weeks after discontinuation of treatment

Study	Age	Patients	Study design	Primary diagnosis	Outcome	Maintenance dose of protein	Notifications
Vickery 2017¹⁹⁶	9mo-3y	37 (20+17) 300 mg dose: 20 3000 mg dose: 17	300 vs 3000 mg arms randomized	OFC	29/37 (78%) achieved 4-week-SU [in 300-mg arm, 17/20 85%; in 3000-mg arm, 12/17 71%]	300 mg or 3000 mg	4-week-SU, target dose comparison, youngest patients
MacGinnitie 2017¹⁹³	6-19y	37 (29+8 placebo)	OIT+omalizumab vs OIT+placebo	DBPCFC (cumulative threshold ≤88 mg)	23/29 (79%) tolerated 2000 mg peanut protein 6 weeks after stopping omalizumab	2000 mg	

2.9 IMMUNOLOGICAL CHANGES DURING IMMUNOTHERAPY

When desensitization or tolerance develops, changes take place at both the cellular and the humoral level. Similar mechanisms are present in natural tolerance development as well as in immunotherapy. ²²²

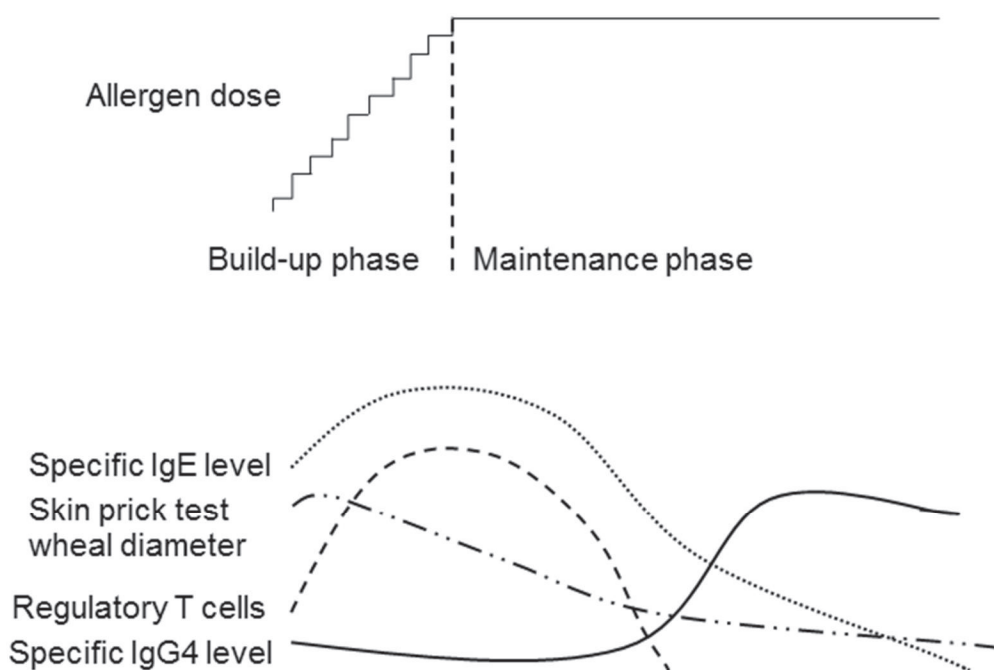


Figure 1. Oral immunotherapy and immunological changes. Adapted from Nowak-Węgrzyn and Albin ¹⁷⁰ with permission of the copyright holder © 2014 John Wiley & Sons Ltd.

Peanut immunotherapy leads to suppression of basophil activation. The upregulation of basophil surface markers shows suppression in patients during active OIT. ^{223, 190, 189}

The changes in allergen-specific IgE levels have been widely documented,²²⁴ whereby they increase initially but decrease in the long-term. In successful patients, IgE levels decrease more than in treatment failures.¹⁸⁹ Irrespective of the change in the amount of specific IgE, the epitope repertoire of IgE broadens during peanut immunotherapy in many patients.²²⁴ Continuous allergen exposure leads to an increase in the allergen-specific IgG4 level.¹⁸⁹ In B cell class switch recombination, the DNA encoding for IgG4 is upstream of the DNA encoding for IgE and is deleted during the class switch to IgE; thus, a switch back to producing IgG4 is not possible.²²⁵ How IgE production shifts to IgG4 is unclear, but it probably involves anergy or the deletion of IgE-producing B cells and an increase in IgG4-producing B cells.²²⁶ In general, the production of IgG4 requires frequent exposure to the allergen. IgG4 is a weak antibody as far as activating effector cells are concerned, but it does have high affinity and can therefore act as a blocking antibody. Both IgE and IgG4 production are induced by IL-4, but IL-10 and T regulatory cells may be crucial in favor of IgG4.²²⁵ IgG antibodies act through FcγRIIb inhibitory receptors in suppressing IgE-mediated reactions.²²⁷ A greater increase in IgG4 may be associated with better outcomes in immunotherapy;^{190, 228} however, epitope specificity seems important in immunotherapy-induced IgG4.²²⁸ In birch pollen immunotherapy, IgE and allergen-specific immunotherapy-induced IgG4 recognize similar Bet v 1 epitopes, which in turn determines if cross-protection occurs.^{229, 173, 230} Birch pollen immunotherapy can induce Bet v 1-specific IgG4 that cross-reacts with related food allergens and competes with the IgE of similar epitopes.²³¹ In addition to changes in IgE and IgG, after peanut OIT, levels of serum IgA increase.²²⁷ An IgA increase has been shown to occur also after milk²³² and egg OITs,²³³ and after sublingual peanut immunotherapy, allergen-specific IgA levels increase in the saliva.²³⁴

3. AIMS OF THE STUDY

The aims of this thesis are to study the following:

- 1) Nut allergy diagnostics by evaluating:
 - nut sensitizations and their clinical relevance in a birch-endemic area **(Study I)**
 - IgE microarray profiles in peanut-challenged patients, and assessing nut-avoidance diets **(Study IV)**
- 2) Peanut oral immunotherapy, by assessing:
 - the efficacy and safety of peanut oral immunotherapy **(Study II)**
 - antibody changes in IgE microarray and singleplex measurements during immunotherapy **(Study III)**

4. MATERIALS AND METHODS

4.1 PATIENTS

Several study populations were used in this thesis for assessing both nut allergy diagnostics and oral immunotherapy for peanut allergy.

A large register-based study population was gathered in Helsinki to study nut allergy diagnostics from the perspectives of birch pollen sensitization and cross-reactivity. To compare two distinct geographical areas, we gathered data on a smaller study population in northern Finland, Lapland Central Hospital.

Another facet of this thesis was an interventional study, which included a population of children and adolescents referred to the hospital due to the suspicion of peanut allergy. These patients underwent an oral peanut challenge, thereby offering a perspective on the gold standard of food allergy diagnostics. Furthermore, a subgroup of challenged patients either received oral immunotherapy for peanut allergy or continued to avoid peanut. This subgroup served in assessing of the safety and efficacy of immunotherapy and the evaluation of antibody changes.

4.1.1 REGISTER STUDY AND INTERVIEWS

In order to study birch pollen sensitization and nut cross-reactivities, **(Study I)** we gathered data on skin prick tests (SPTs) from the Skin and Allergy Hospital database, which includes all SPTs conducted in the hospital, and we selected tests from 1997 until the study initiation year of 2013. We selected all SPTs conducted for birch pollen and formed a study population based on these individuals. For these subjects, we searched the SPTs for any nut species and formed a subpopulation of both birch pollen- and nut skin prick-tested individuals. The included nut species were walnut, pecan, pistachio, cashew, coconut, Brazil nut, macadamia, almond, hazelnut, and peanut. If an individual was tested more than once, we included only the most recent result.

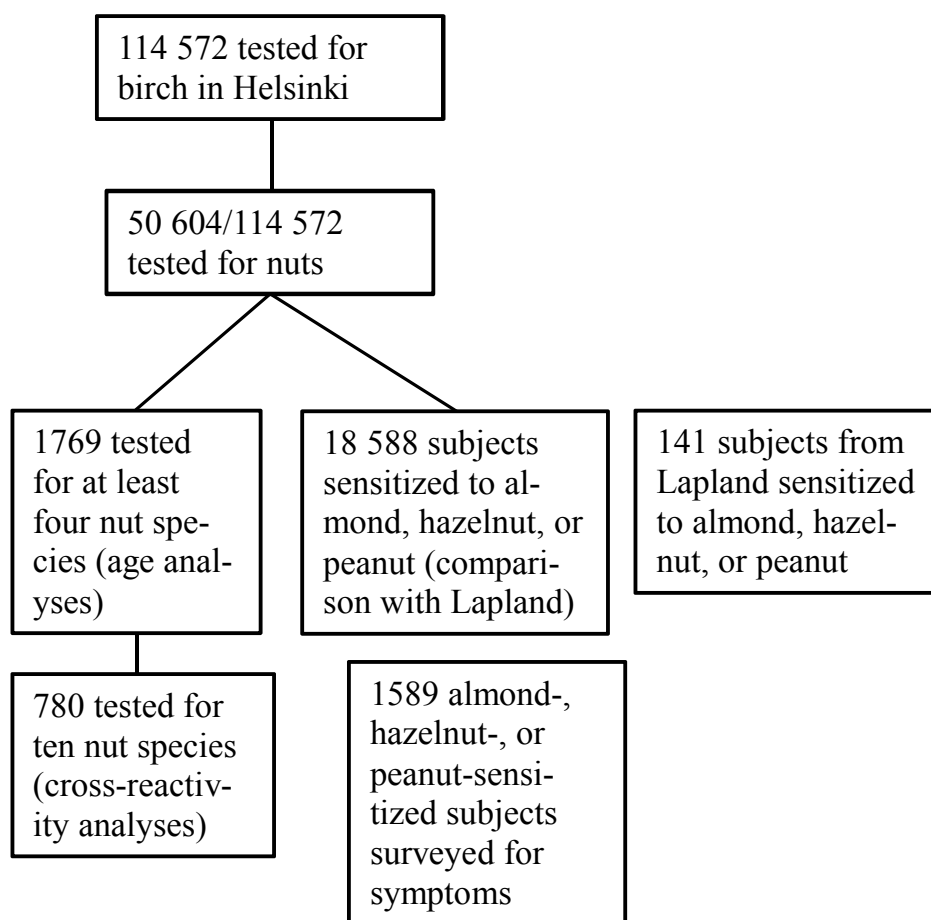


Figure 2. Flow chart of patients in study I. Adapted from study I with permission of the copyright holder © EAACI and John Wiley and Sons A/S. Published by John Wiley and Sons, Ltd.

To assess age differences relating to sensitization, subjects with results available for at least four nut species were included. This was done because subjects with only three nut species examined were usually tested for hazelnut, almond, and peanut as a part of the vegetable and spice test panel. The indication for testing the vegetable and spice panel involves mainly the screening of any food sensitization without specific suspicion of a nut allergy. In order to focus the study on individuals with a suspected nut allergy, we focused on those that had at least four nut species examined. For the age group analyses, we formed three groups (under 5 years, 5-15 years, 16 years and older) to assess the

development of sensitization during childhood and adolescence. In cross-reactivity analyses, we included individuals who had all ten nut species examined.

In addition to data from the Skin and Allergy Hospital, we gathered data from northern Finland, Lapland Central Hospital (Rovaniemi). From an archive of skin prick tests, we selected subjects who were sensitized to hazelnut, almond, or peanut and had also been tested for birch pollen. Furthermore, we studied the overall prevalence of birch sensitization in Lapland in a separate population of 359 subjects tested for birch pollen during 2011–2012.

We assessed the differences in birch pollination between the two study regions based on pollen counts provided by the University of Turku, Aerobiology Unit (Turku, Finland). Data included years 1995–2012 in Helsinki and years 2002–2012 in Rovaniemi.

4.1.2 INTERVENTION STUDY

Nut allergy diagnostics were studied further in a smaller population of children and adolescents referred to the Skin and Allergy Hospital pediatric department, due to a suspicion of peanut allergy. The patients, aged 6–18 years, were recruited between 2011 and 2013. Of the patients, 102 were double-blind placebo-control challenged to peanut and thereby formed a group where the characteristics of the challenges and details of other nut consumption could be assessed (**Study IV**) (Figure 3). Previously, a separate study, concentrating purely on peanut allergy component diagnostics, was published based on this population.¹⁰⁹

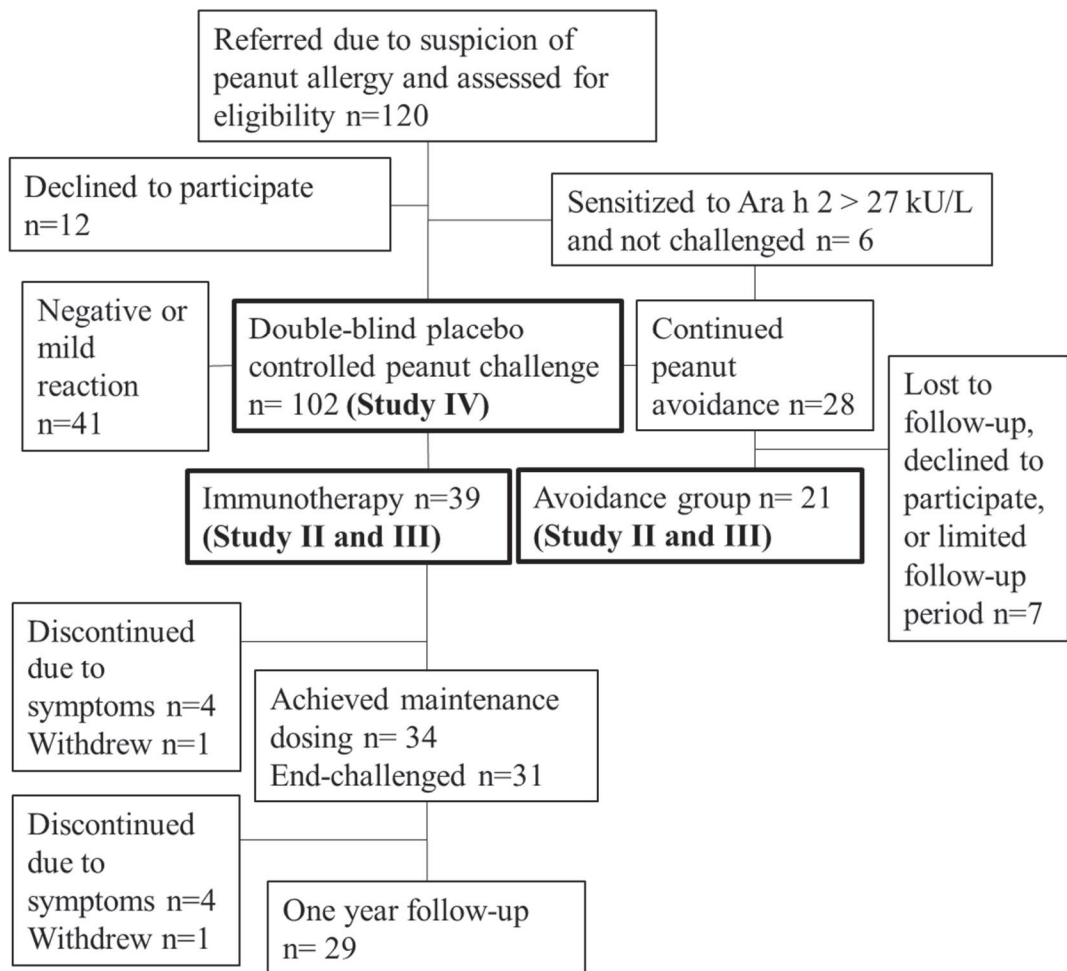


Figure 3. Flow chart of patients in studies II-IV. Adapted from study II with permission of the copyright holder ©2016 Foundation Acta Pædiatrica. Published by John Wiley & Sons Ltd.

Of the challenged 102 patients, a subgroup of 54 and an additional six non-challenged patients participated in the study of peanut oral immunotherapy. Of this group, 39 patients started immunotherapy and 21 continued an avoidance diet. **(Study II)**

In order to study antibody changes during immunotherapy, we included immunotherapy patients with available serum samples (n=58). **(Study III)**

4.2 SKIN PRICK TESTING

Our skin prick testing method utilized raw nuts which were ground up unpeeled and then mixed with 0.9% saline. A commercial extract (ALK-Abelló, Hørsholm, Denmark; or Allergopharma, Reinbek, Germany) was used for birch pollen testing. Histamine hydrochloride (10 mg/ml) and the allergen solvent were used as positive and negative controls. The skin was pricked with a single-headed metal lancet on the volar forearm. With nuts, a prick-to-prick method was used. Results were read after 15 minutes. Wheal sizes of 3 mm or larger were documented in millimeters, and wheals smaller than 3 mm were documented as zero. The results for Lapland Central Hospital were reported as 0 through 4+ before mid-2009, and thereafter a similar method as in the Skin and Allergy Hospital was used. We considered a wheal size of at least 3 mm or 1+ positive.

4.3 INTERVIEWS ON THE CLINICAL SIGNIFICANCE OF SKIN PRICK POSITIVITY

We assessed the clinical significance of skin prick positivity by interviewing sensitized patients. A survey of symptoms was performed with 1,307 subjects sensitized to hazelnut, with 1,159 to peanut, and with 1,099 to almond. The survey was conducted during 2006- 2014 at the Skin and Allergy Hospital according to Haahtela et al.⁹⁷ All age subjects who had at least a 3 mm wheal reaction for any of the three nuts (almond, hazelnut, or peanut) were eligible for the study. The nurses interviewed the study subjects on site, using a structured interview. Symptoms were categorized as conjunctival symptoms, otorhinolaryngological symptoms (sneezing, rhinitis, congestion, pruritus of the throat or ears), symptoms affecting the lower airways, skin symptoms, contact urticaria, gastrointestinal symptoms (diarrhea or vomiting), and anaphylaxis. Anaphylaxis was defined according to the 2006 criteria provided by Sampson et al.²³⁵

4.4 EVALUATION OF POTENTIAL IMMUNOTHERAPY PATIENTS AND THE CHALLENGE PROCEDURE WITH PEANUT

In addition to patient history, laboratory and skin prick tests (SPTs) were conducted. The panel of IgE measurements included serum total IgE, peanut- and hazelnut-specific IgE, and component-specific IgEs for peanut, hazelnut, and cashew. The SPT panel included aeroallergens (birch, timothy, mugwort, cat, dog, mite *Dermatophagoides pteronyssinus*, mold *Cladosporium herbarum*)

and foods (egg, wheat, gliadin, cow's milk, peanut, hazelnut, almond, cashew, pecan, walnut, pistachio, Brazil nut, macadamia, coconut, sesame, linseed, pine nut, poppy seed and sunflower seed). The patients and their families filled in questionnaires on their previous usage of nut and seed species and on food-specific quality of life.

A double-blind, placebo-controlled peanut challenge was performed on two separate days. The challenge included four allergen doses: 5-50-200-1000 mg of peanut protein resulting in a 1255 mg cumulative dose. The 1255 mg dose corresponds approximately to six whole peanut kernels. The doses were administered in 30-minute intervals. The challenge preparation was made up of crushed unroasted peanut mixed with blueberry powder and banana chips. The matrix served as a placebo. The challenge was considered positive only in the case of objective symptoms.

4.5 IMMUNOTHERAPY INTERVENTION

All patients enrolled in oral immunotherapy had a moderate-to-severe reaction at the baseline DBPCFC. Six patients in the control group were not challenged but had high Ara h 2 (range 27.8 to 365 kU/L). Patients who had poor asthma control (forced expiratory volume in one second, FEV1 <80%), any major chronic illness, or ongoing immunotherapy were excluded. The OIT group underwent the eight-month OIT build-up phase, in which the amount of peanut protein increased from 0.1 mg to 800 mg (approximately four whole peanuts) per day. As an OIT preparation, roasted defatted peanut flour (50% protein, 12% fat [Byrd Mill, VA]) was used mixed with soy- and milk-free margarine (Keiju 70%, Raisio, Finland). From week 20, patients used whole peanuts, which could be raw or roasted according to patients' preferences. Patients took antihistamines daily during the build-up phase. Adverse symptoms were assessed on scheduled hospital visits and with additional phone calls and check-ups, when needed. The patients were provided with an adrenalin autoinjector, antihistamines, and prednisolone in the case of adverse reactions.

After reaching the maintenance dose of 800 mg peanut protein, the DBPCFC was repeated. Desensitization was defined as the ability to pass the DBPCFC with 1255 mg peanut protein. In the control group, failure to desensitize was confirmed by the peanut challenge, or persistent high serum IgE to Ara h 2. We calculated the amount of peanut protein ingested during OIT, using the dose increment protocol and the individual treatment days.

During the maintenance phase, the patients took four peanuts daily or three to four times per week.

4.6 IGE MEASUREMENTS

Serum IgE measurements in this thesis were conducted both with singleplex ImmunoCAP assays and multiplex ImmunoCAP ISAC microarrays (Thermo Fisher Scientific, Uppsala, Sweden). Sensitization was defined as ≥ 0.35 kU/L in IgE ImmunoCAP and ≥ 0.3 ISU-E in ISAC. The detection limits were 0.1 kU/L for IgE in ImmunoCAP and 0.3 ISU-E in ISAC. Serum IgG4 was measured only with singleplex ImmunoCAP assays with 0.07 mg/L as detection limit.

IgE measurements were conducted in all patients who participated in the intervention study. We measured serum total IgE, and specific IgE and specific IgG4 antibodies to peanut and Ara h 1, 2, 3, 8, and 9, and hazelnut Cor a 14 and cashew Ana o 3 using a singleplex immunoassay. In immunotherapy patients, the IgE levels were measured in the beginning of the treatment, after reaching the maintenance phase and after receiving one year maintenance treatment.

(Study II)

IgE and IgG4 antibodies to Ara h 6 were measured with an experimental ImmunoCAP test in Thermo Fisher Scientific, Uppsala, Sweden. We analyzed the ratio of IgE and IgG4 to peanut allergen components and their changes during the immunotherapy. **(Study III)**

In all of the 102 challenged patients, we analyzed the general IgE sensitization profiles on ISAC microarrays and assessed the importance of all 112 allergens included in the array in predicting the challenge result. Furthermore, we assessed family-reported avoidance of nut species and compared this information to sensitizations examined with skin prick tests and sensitizations to species-specific allergens present in the microarray. Species-specific measurements in the ISAC microarray and in singleplex ImmunoCAPs included peanut, hazelnut, cashew, pistachio, Brazil nut, pecan, walnut and sesame seed. **(Study IV)**

4.7 AIRWAY MEASUREMENTS

As part of the immunotherapy study, we measured airway effects. A methacholine challenge test was performed before and after the build-up phase in the immunotherapy patients, in order to measure bronchial hyperresponsiveness. The provocative dose causing a 20% decrease in forced expiratory volume in one second (PD₂₀FEV₁) was measured. In addition, the fractional concentration of exhaled nitric oxide (FeNO) was measured, utilizing an online method using an Ecomedics CLD 88 analyzer.²³⁶ **(Study II)**

4.8 STATISTICAL ANALYSES

4.8.1 REGISTER STUDY

The sample size in the register study (**Study I**) was based on available skin prick tests in the two hospitals during the study years and the available results from the symptom interviews. We analyzed the data with chi-square and Fisher's exact tests for dichotomous variables. A Spearman's rank-order correlation was used for continuous and categorical variables. Hierarchical clustering with Euclidean distance and the method of average linkage were used to create an analysis of the cross-reactivities of all ten nut species. Trends in sensitization according to age group were analyzed with the linear-by-linear association test and a Poisson regression. All tests were done two-sided, and the P value <0.05 was considered significant. Statistician, PhD Hannu Kautiainen analyzed the proportion of symptomatic patients with binomial logistic regression.

4.8.2 INTERVENTION STUDY

The sample size of 102 patients, who were double-blind placebo-control challenged to peanut, was based on a previous study on peanut allergy diagnostics. They were a consecutive sample of referred patients.¹⁰⁹ (**Study IV**) We used a Mann-Whitney-U test to compare individual allergens in ImmunoCAP ISAC and singleplex ImmunoCAPs. In multiple pairwise comparisons, a P value of <0.0004 was considered significant, based on the Bonferroni method, as the comparisons included two allergens from ImmunoCAP measurements and 112 from ImmunoCAP ISAC, i.e. totaling 114 pairwise comparisons. In other analyses, a P value <0.05 was considered significant.

The correlation of the IgE level, the challenge threshold dose, and skin prick wheal size was analyzed with a Spearman's correlation, while dichotomous variables were analyzed with Fisher's exact test. Statistician Patrik Dykiel, from Thermo Fisher Scientific, conducted a random-forest analysis of the importance of microarrayed allergens on predicting the challenge result.

PhDs Joost Westerhout, Marty Blom, and Ben Remington from the Netherlands research organization TNO FARRP, analyzed the threshold doses of positive challenges. For the threshold distribution curve, individual discrete and cumulative no observed adverse effect levels (NOAELs), and lowest observed adverse effect levels (LOAELs), were established in all challenge-positive patients. The discrete and cumulative individual NOAELs and LOAELs were analyzed through interval-censoring survival analysis and fitted to population threshold distribution curves from which the eliciting doses could be determined, and those predicted to provoke reactions in 5%, 10%, and 50% of the population were estimated using the Log-Normal, Log-Logistic, and

Weibull parametric models.⁹³

In the immunotherapy study (**Studies II and III**), the main outcome measure was the number of desensitized patients in each group. Desensitization was defined as the ability to pass the DBPCFC with 1255 mg peanut protein, after the build-up phase. We calculated that the sample of 39 patients in the OIT group and 21 in the avoidance group would provide 93% power to detect a significant difference in the rate of desensitization between the study groups at a 5% two-sided significance level, when assuming that 20% of the avoidance patients would recover spontaneously and 60% of the OIT group would become desensitized by the treatment protocol.

Prof. Dario Greco, from Helsinki University, Institute of Biotechnology, conducted the statistical analysis of ISAC microarrays and IgG4-to-IgE ratios. We filtered the results of the ISAC microarray so that the measured value of an allergen had to be at least 0.3 in at least 20 OIT patients and in ten avoidance patients (50% +1), in at least one of the four groups (OIT group pre, OIT group post, avoidance group pre, avoidance group post), in order for the allergen to be included in the analysis. A linear model was fitted to examine the effects of the treatment, response, subject, gender, age, and season. Furthermore, we used eBayes for pairwise comparisons of interest. P values were corrected using the Benjamini-procedure. For the IgG4-to-IgE ratios, we converted IgE to mg/L with a conversion factor of 1 kU/L = 0.0024 mg/L. R software with the limma package was used to analyze the microarray and IgG4-to-IgE ratio results. For other analyses, we used Fisher's exact test and the Mann-Whitney U test to compare two groups, while the Wilcoxon signed rank test was employed to compare repeated measurements.

In all studies, we used IBM SPSS Statistics software (IBM, Armonk, NY, USA), and in studies I, III, and IV we also used R program 3.0.2 (R Project for Statistical Computing, <http://www.r-project.org/>).

4.9 ETHICS

The ethics committee at Helsinki University Hospital and Lapland Central Hospital approved the studies. In addition, the study was registered at Clinicaltrials.gov (NCT01502878). Each patient and either one of her/his parents signed a written informed consent to participate in the intervention study.

5. RESULTS

5.1 NUT ALLERGY DIAGNOSTICS IN A BIRCH-ENDEMIC AREA

5.1.1 SKIN PRICK TESTS AND CROSS-REACTIVITIES

During the years 1997-2013, a total of 114,572 individuals were tested for birch pollen. Baseline characteristics of the study population are presented in Table 5, below.

Table 5. Baseline characteristics of the 114,572 individuals skin prick tested for birch pollen. (Study I)

Age, median (interquartile range)	28 years (11-45)
Positive skin prick test for birch pollen, n (%)	35 976 (31.4% [95% CI, 31.1-31.7%])
Tested for at least one nut species, n (%)	50 604 (44.1% [95% CI, 43.9 - 44.5%])

CI; confidence interval

Sensitization to nut species was more common in subjects who were simultaneously sensitized to birch pollen ($P < 0.001$ difference for all nut species). Hazelnut had a 15.9-fold prevalence in birch-positives compared to -negatives. Of hazelnut-sensitized subjects, up to 84% were concurrently sensitized to birch pollen. (Study I, Figure 2.)

The correlation with birch wheal size was strongest with hazelnut (Spearman $\rho = 0.84$, $P < 0.001$), almond ($\rho = 0.74$; $P < 0.001$), and peanut ($\rho = 0.61$, $P < 0.001$) wheals.

In the symptom surveys, the proportion of symptomatic patients was higher in line with increasing skin prick wheal size. (Study I, Figure 4.)

The majority of patients experiencing symptoms from nuts (57 to 66%) reported having only otorhinolaryngological symptoms. The details of the symptoms are shown in Table 6.

Table 6. Details of symptoms reported by nut-sensitized subjects.

Type of symptoms n (%)	Peanut (n=1159)	Hazelnut (n=1307)	Almond (n=1099)
Otorhinolaryngological (sneezing, rhinitis, congestion, pruritus of the throat or ears)	601 (52)	766 (59)	499 (45)
Otorhinolaryngological only	421 (36)	518 (40)	378 (34)
Lung (cough, mucus, wheeze, dyspnea)	170 (15)	231 (18)	105 (10)
Skin (eczema, urticaria)	102 (9)	114 (9)	68 (6)
Gastrointestinal (vomiting, diarrhea)	51 (4)	24 (2)	12 (1)
Eyes (watering, pruritus, oedema)	29 (3)	27 (2)	14 (1)
Contact urticaria	17 (1)	27 (2)	15 (1)
Anaphylaxis	19 (2)	8 (1)	2 (0.2)

The hierarchical cluster analysis of nut species and birch pollen showed that birch formed a cluster with hazelnut, almond, and peanut. Cashew and pistachio formed a pair as well as pecan and walnut. (Study I, Figure 5.)

Sensitization prevalence for all nut species decreased with age in children and young adults in those subjects that were not sensitized to birch pollen, whereas in the birch-positives, nut sensitization prevalences seemed to increase in adolescence. (Study I, Figure 6.)

When comparing the two geographical areas, the prevalence of birch pollen sensitization among all the tested subjects was lower in Helsinki than in Lapland (24.0% [95% CI, 19.6–28.4%] vs 31.4% [95% CI, 31.1–31.7%], $P = 0.003$). In the nut-sensitized populations, no difference in birch sensitization prevalence was detected in the two areas.

5.1.2 IGE MICROARRAY PROFILES AND AVOIDANCE DIETS

Analysis of the IgE microarray profiles in peanut-challenged patients showed that the most important allergens for predicting peanut challenge result were Ara h 2 and Ara h 6. (Study IV, Figure S3.)

The cumulative threshold dose for the positive challenge reaction correlated (Spearman's rho) with the IgE levels to Ara h 1 (-0.49), 2 (-0.51), 3 (-0.54), and 6 (-0.55), but not with Ara h 8 and 9.

PR-10 sensitization was common (90% for Bet v 1) throughout the whole group, but it did not differ between the challenge positives and negatives. (Study IV, Figure 2.)

The avoidance habits of peanut-sensitized children and adolescents showed that

up to 52-96% avoided some tree nut species, and only 27-44% were sensitized to the respective species-specific proteins in the microarray. (Study IV, Figure 3.)

5.2 ORAL IMMUNOTHERAPY TO PEANUT

5.2.1 EFFICACY AND SAFETY

Peanut OIT resulted in desensitization in 33/39 patients in the treatment group, and 26/39 were able to tolerate 1255 mg peanut protein in the re-DBPCFC after reaching the maintenance dose. Of the 39 patients, 31 were able to undergo the re-DBPCFC according to the study protocol.

The tolerated peanut protein dose increased from a median 5 mg (range 0–255 mg) to 1255 mg (255–1255 mg) ($P < 0.001$).

The median serum IgE for whole peanut extract, and Ara h 1, 2, 3, 8, and 9 remained at the same level before and after the OIT build-up phase, whereas serum IgG4 concentrations for the whole peanut extract, and Ara h 1, 2, 3, 6 and 8 increased significantly. (Study II, Table S2.) After one year of maintenance treatment, the serum IgE levels for Ara h 1, 2, 3, 8, and 9 decreased in the 29/39 patients that continued the treatment. (Study II, Table S2.)

Of the OIT patients, 33/39 had available results for the methacholine challenge test. Forced expiratory volume in one second (FEV1) as a percentage of predicted was unaffected during the OIT build-up phase ($P = 0.14$). The number of patients with moderate or severe hyperresponsiveness, i.e. a cumulative methacholine dose inducing a 20% reduction in FEV1 under 600 μg ($\text{PD}_{20}\text{FEV1} < 600 \mu\text{g}$), did not change. However, an increase in the methacholine dose ($\text{PD}_{20}\text{FEV1}$), from 810 μg to 1451 μg , was observed ($P = 0.27$). Fractional exhaled nitric oxide (FeNO) showed no change, though the number of patients with an increased FeNO level (z-score > 1.96 or > 25 ppb) decreased from 22 to 14 patients ($P = 0.08$). (Study II, Table 3.)

Of the OIT patients, 30/39 (77%) reported adverse symptoms during the build-up phase, with the majority being mild oral symptoms that needed no medication. Five patients sought emergency medical care because of symptoms related to the treatment, and one of these five used an adrenaline auto-injector. In the long term, which included a median 31 months (range 1.6-52) of treatment in the build-up and maintenance phases, an additional six OIT patients sought medical treatment and three patients needed adrenalin for a peanut reaction.

Food allergy-related quality of life improved from median 57 (range 1–109) to

40 (15–101) points ($P = 0.03$) in the 27 patients who had available results from the questionnaire. The 26 studied parents showed no change in their quality of life.

5.2.2 ANTIBODY CHANGES

Changes in IgE levels in microarrays before and after OIT build-up phase occurred only in Ara h 2 and Ara h 6. IgE for Ara h 2 decreased significantly from a median 39 ISU-E (range 1.0–176) to 6 ISU-E (1.2–86), and for Ara h 6 from 35 ISU-E (1.4–157) to 4.9 ISU-E (1.0–37) ($P < 0.0001$). (Study III, Figure 1.)

IgG4-to-IgE ratios for Ara h 1, 2, 3, 6, and the whole peanut extract increased significantly during treatment, whereas ratios for Ara h 8 and Ara h 9 remained stable. (Study III, Figure 2.)

The increase in IgG4-to-IgE ratios for Ara h 2, 3, 6, and the whole peanut extract were associated with the cumulative amount of peanut protein ingested during treatment: Ara h 2 (Spearman $\rho = 0.50$, $P = 0.001$), Ara h 6 ($\rho = 0.36$, $P = 0.026$) ($n = 38$), Ara h 3 ($\rho = 0.43$, $P = 0.007$), and whole peanut extract ($\rho = 0.47$, $P = 0.002$).

6. DISCUSSION

This thesis includes studies on sensitization, cross-reactivities, and laboratory diagnostics of nut allergies. We have studied oral immunotherapy for peanut allergy and its effects on underlying sensitizations and antibody profiles. Furthermore, we assessed avoidance of nut species in peanut-sensitized patients and compared their avoidance diets to species-specific sensitization profiles.

6.1 METHODOLOGICAL CONSIDERATIONS

6.1.1 STUDY POPULATIONS

In the cross-sensitization study we included all patients that were skin prick tested for birch pollen at the Skin and Allergy Hospital during the chosen study years 1997-2013. This resulted in a very large sample size, at over 100,000 individuals. The tested individuals had all had some clinical indication for skin prick testing, i.e. suspicion of an allergy or atopy. This makes our results generalizable to patients that need assessment of sensitizations and advice on the very common finding of nut sensitization. The findings of our study will help to assess sensitized patients in primary as well as in specialist care centers. Our study population in Rovaniemi was markedly smaller than the Helsinki population, because the general population of Lapland is smaller and, in addition, we were not able to include all patients skin prick tested to birch pollen during the study years, because the archive had to be searched and saved manually. The Rovaniemi study population of nut-sensitized patients included mainly hazelnut-, almond-, and peanut-tested patients. Other species were very infrequently tested, which limited the geographical comparison to only these commonly tested nuts. We assessed overall birch pollen sensitization rates in Rovaniemi from a separately collected population of patients that were tested during the time when the results were reported in millimeters. For nut sensitization, we also included qualitative results, which limited us to studying sensitization only from a qualitative point of view.

Our study is limited by the fact that we assessed sensitizations only. Clinical relevance was assessed only in a subgroup of patients and was based on patient-reported symptoms and no challenges were conducted. Some patients may possibly have experienced more severe symptoms when challenged to high doses of nut protein, however, any severe reactions were not confirmed by the challenge, either. Furthermore, our study population included subjects with the clinical suspicion of birch and nut allergies, and our data are thus subject to

overestimation of these co-sensitizations. Moreover, the findings of age-related changes can be regarded only as indicative and may be skewed by any possible differences in patient selection or changes in population rather than at an individual level. Our research extends to the year 1997 and was designed to study cross-sensitizations in skin prick tests. We were limited to skin prick tests, and the lack of serum samples, and due to the large sample size we, we were not able to analyze serum-specific IgE. In clinical use, however, component-resolved diagnostics is adapted to discriminate between cross-sensitization and true allergy.

In addition to skin prick testing, we studied the diagnostics of nut allergy in patients who were double-blind placebo-controlled challenged to peanut. This enabled us to compare laboratory measurement diagnostics to the gold standard double-blind placebo-controlled challenge. Our population was selected based on sensitization to peanut either in skin prick tests or in an in vitro IgE test for the whole peanut extract. This makes our results generalizable to patients referred to specialist centers or examined in a primary health care setting and who are peanut-sensitized. Our patients had either experienced symptoms from peanut previously or had never ingested peanut and were unaware of their allergy status. This setting reflects true nut allergy diagnostic dilemmas accurately, as many children, especially younger ones, have never ingested specific nut species.

Peanut oral immunotherapy was studied in severely allergic patients. The allergy was diagnosed with a double-blind placebo-controlled peanut challenge, and our inclusion criteria for OIT required patients to have at least a moderate-to-severe reaction in the challenge, in order to be eligible for immunotherapy. Thus, our patients had a true and severe peanut allergy, and patients with only pollen-cross-reactive peanut sensitization were excluded. This study was designed to assess the efficacy and safety of peanut oral immunotherapy in moderate-to-severely allergic children with even anaphylaxis, as they would benefit the most from this treatment.

A major limitation of our study was that the study groups were not randomized but families chose themselves whether or not they wanted to have immunotherapy. Also, subgroup analyses according to outcomes were limited in this study, because the study was powered to show the efficacy of desensitization. Thus, we were unable to unravel factors that would predict success in OIT.

In the study of antibody changes during immunotherapy, we included all patients that received the intervention and control patients that continued to avoid peanut, i.e. the traditional treatment for nut allergy. Two patients from the avoidance group were excluded because of an unavailable serum sample. The sample size in peanut oral immunotherapy was based on the primary outcome, which was the ability to pass the end-challenge after achieving the

maintenance phase. Thus, we were limited to this sample size when assessing our secondary outcomes.

6.1.2 CHALLENGES

Peanut challenges were conducted in a double-blind placebo-controlled fashion, which is the gold standard of food allergy diagnostics. The cumulative 1255 mg protein dose is sufficient to exclude reactivity to accidental amounts of peanut. The study challenges were conducted according to PRACTALL guidelines,¹⁵⁶ which makes them generalizable to other study centers. The lowest dose of peanut protein in our challenge protocol was selected based on clinical demands and was set to 5 mg. Lower starting doses would have enabled us to examine the threshold dose for the Finnish population more accurately, however, our threshold results were in line with published thresholds from other populations.

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6.1.3 IGE MEASUREMENTS

IgE was measured both in singleplex ImmunoCAP and in multiplex microarray ImmunoCAP ISAC. In singleplex measurements, serum samples were diluted if the results exceeded the 100 kU/L limit, in order to measure even the highest IgE levels in our patients, some of whom had IgE levels counting several hundreds. We were able to study IgG4 in ImmunoCAPs as well, and this method was not a routine standard at the time of the study.

In the IgE microarray ImmunoCAP ISAC, we were able to screen our patients in relation to 112 allergens from a large variety of allergenic sources and protein families. As the microarray is semi-quantitative, we were unable to compare our results directly from the singleplex ImmunoCAPs to the microarray.

6.1.4 AIRWAY MEASUREMENTS

Airway inflammation in patients receiving peanut oral immunotherapy was measured by examining fractional exhaled nitric oxide and airway hyperresponsiveness by methacholine challenges. The measurements were conducted before treatment and approximately one month after achieving the maintenance phase, i.e. after the build-up phase. Fractional exhaled nitric oxide is recommended for monitoring airway inflammation in patients with asthma²³⁷, while the methacholine challenge is recommended for testing airway hyperresponsiveness.²³⁸ In our study, patients' asthma treatment was conducted according to clinical needs. As asthma is a risk factor for severe reactions in food-allergic reactions, we assessed possible asthma symptoms in all study visits. None of our patients was excluded from the study due to poor asthma control.

6.2 DISCUSSION OF THE MAIN RESULTS

6.2.1 NUT ALLERGY DIAGNOSTICS IN A BIRCH-ENDEMIC AREA—SKIN PRICK TESTING, IGE MICROARRAY PROFILES, AND AVOIDANCE DIETS

6.2.1.1 *Sensitization to nuts in a birch-endemic area, symptoms, and cross-reactivities*

The prevalence of nut sensitization was up to 84% in birch-sensitized individuals. With higher levels of birch sensitization, an increasing proportion of tested subjects were sensitive to multiple nuts.

Hazelnut is one of the most common food ingredients from which birch-allergic individuals report experiencing symptoms.⁵⁶ In the Finnish population, birch sensitization is directed mainly toward the pathogenesis-related 10 (PR-10) protein Bet v 1⁵⁵, which in turn drives other sensitizations toward PR-10 proteins in other species.⁵⁸ Among nut species, PR-10 proteins are characterized in hazelnut, almond, peanut⁵⁹, and walnut.⁴⁶ Our study results showed that hazelnut, almond, and peanut cross-reacted with birch pollen the most.

In the symptom survey, most subjects reported experiencing either no or only mild symptoms, which were mainly otorhinolaryngological, i.e. corresponding to oral allergy syndrome. Previous clinical studies on allergenic components in nuts show that sensitization solely to PR-10 proteins Cor a 1 in hazelnut and Ara h 8 in peanut causes mild or no symptoms^{119, 70}, whereas sensitization to seed storage proteins Cor a 9 and 14 and Ara h 2 and 6 causes severe symptoms.^{123, 111} The mild symptom profile of our study subjects links to the fact that nut sensitization in birch-endemic areas is mostly pollen-related and without severe symptoms.

Age group analyses showed that young children that were not sensitized to birch pollen were already sensitized to nuts, which is in accordance with the allergic march, in that sensitization to foods emerges earlier than pollen sensitization.²³⁹ In our study, this non-birch-induced sensitization to nuts decreased with age. The phenomenon of decreasing nut sensitization during the first years of life was also demonstrated in the study by Peters et al., which showed that 22% of children lost their peanut allergy by 4 years of age and decreasing skin prick wheal size predicted the development of tolerance.³⁸ Our study subjects that were sensitized to birch, however, exhibited no decrease in sensitization to most nut species during childhood and adolescence. We hypothesize that this is due to the increase in their pollen sensitization and their subsequent cross-sensitization to nuts. Cohort studies on peanut allergy show that asymptomatic sensitization increases until adulthood, but clinical reactivity

increases only during early childhood.^{240, 241} Correspondingly, this suggests that asymptomatic peanut sensitization is induced by pollen sensitization. A Europe-wide study showed that pollen-related allergy to hazelnut was more common in adults than in children.⁷²

In our co-sensitization analyses, pecan and walnut, as well as cashew and pistachio were the two nut pairs that showed the highest co-sensitization. These species originate from the same botanical families, i.e. pecan and walnut from the family Juglandaceae, and cashew and pistachio from Anacardiaceae.²⁴²

Previous studies on skin prick tests and specific IgE show similar findings in relation to these nut species.^{243, 106, 244} In the hierarchical cluster analysis, a similar result to the correlation analyses was present, in that almond, hazelnut, and peanut grouped with birch pollen.

We were able to study cross-sensitizations in two distinct areas of Finland. The distribution of birch pollen differs in the southern and the northern regions of the country, which enabled us to compare sensitizations in areas with differing pollen exposures. In the overall population tested for birch pollen, sensitization was less common in the north, in accordance with the lower exposure. The very high birch pollen count in southern Finland is markedly higher than in most of Europe and in the USA,^{245, 246} while the Rovaniemi region has birch pollen counts comparable to central Europe.

In the comparison of nut cross-sensitizations, we selected subjects that were skin prick tested for hazelnut, almond, and/or peanut, were sensitized to at least one of these nuts, and had also been tested for birch pollen. In these nut-sensitized subjects from the two regions, concurrent sensitization to birch pollen was equally common. The comparison was limited to hazelnut, almond, and peanut, because these were the most commonly tested nuts and offered an adequate sample size for the comparison. The proportions were highly similar in the two regions, when we compared the rate of hazelnut-sensitized or peanut-sensitized subjects with simultaneously birch-sensitized subjects. Almond showed higher concurrent birch-sensitization prevalence in Helsinki than in Rovaniemi, though this difference failed to reach statistical significance. Hazelnut sensitization was again associated most strongly with birch sensitization.

Previous studies based in molecular allergology showed that peanut and hazelnut allergies have a stronger association with birch pollen sensitization in Northern Europe than in Southern Europe or the USA.^{72, 247, 82} As Rovaniemi has birch pollen counts similar to central Europe, our results are generalizable outside Finland.

Our study showed that subjects with larger skin prick wheal sizes for birch pollen had concurrent sensitization for increasing numbers of nut species. This may reflect a general pattern where patients with stronger reactivity to one allergen react to several allergens in general. On the other hand, this strengthens

the finding that birch pollen sensitization is associated strongly with many nut sensitizations.

6.2.1.2 *Microarray in the diagnostics of peanut allergy, and an assessment of the avoidance diets of nut-allergic patients*

Overall, the sensitization patterns of peanut-challenged patients showed that peanut-allergic and -tolerant patients had a similarly high prevalence of sensitization to PR-10 allergens. Ara h 8 sensitization was very common in our patients, both in peanut-tolerant as well as in -allergic patients. Our finding highlights the fact that Ara h 8 sensitization is pollen cross-reactivity and true peanut allergy is not caused by Ara h 8 sensitization. It is not recommended to test Ara h 8, as it seems not to add any value in differentiating severely allergic from tolerant.²⁴⁸ In line with Ara h 8, the other PR-10 protein sensitizations were similar in the challenge-negative and -positive groups. IgE levels for the PR-10 proteins were slightly higher in the challenge negatives, but this difference failed to reach statistical significance. Strongly PR-10-sensitized patients may be more likely to have been selected for our study population, as they were more probably previously suspected of having true peanut allergy. In addition, mild oral allergy symptoms, due to PR-10 sensitization, may have occurred in these patients and further enhanced the suspicion of true peanut allergy. Thus, in these patients, a controlled challenge for peanut is especially useful in preventing unnecessary avoidance of the nut.

The threshold distribution curve of our 69 challenge positive patients showed that our study population was representative of the generic allergic population. When comparing the curves to a larger, previously published multinational allergen threshold database, no significant population differences were observed.²⁴⁹ However, the starting dose in our challenge protocol was 5 mg, and this dose leads to response in 15-20% of patients, so it was not possible to extrapolate the eliciting doses for 5% and 10%. The calculated values for a 50% eliciting dose were comparable to the values established by Taylor et al.²⁴⁹ For clinical purposes, a starting dose of 5 mg peanut protein is beneficial, as a higher starting dose makes it possible to conduct the challenge in one day. Challenge positives had higher IgE to peanut seed storage proteins, especially Ara h 2 and Ara h 6. IgE levels to the seed storage proteins Ara h 1, 2, 3, and 6 were associated with the challenge threshold doses. Previously, a Danish study showed a weak correlation of Ara h 1, 2, and 3 with the peanut threshold dose.²⁵⁰

Our highly birch-sensitized study population had skin prick positivity to several nut species, and they also reported never having ingested many of the inquired nuts. Coconut, almond, and sesame seed showed lower rates of avoidance, which might be due to an overall higher consumption of these species. Coconut, almond, and sesame are sometimes not considered nuts in a culinary sense, and

a family may consider these species safe to eat despite setting out to avoid all nuts. Coconut has minor cross-reactivity with other nuts and is also botanically distant from the other species.^{251, 252} As sesame seeds are used in many convenience food hamburgers as well as Middle-Eastern and Oriental foods that have become more popular during recent years, their consumption may have increased. The avoidance of Brazil nut and macadamia may be affected by the generally low supply of these species, and families may not identify these species.

An important reason for avoiding all nuts may be that families are afraid of cross-contamination.²⁵³ Identifying different nut species is also difficult²⁵⁴, but unnecessary avoidance may even prevent the development of natural oral tolerance.¹⁴

In IgE measurements of the available seed storage proteins of nuts, we found that sensitization to these proteins was infrequent. Concurrently, sensitization to the whole nut species in skin prick tests was common. The high rate of sensitization in skin prick tests is probably caused by birch cross-reactivity and in most cases does not include sensitization to stable allergens.

6.2.2 ORAL IMMUNOTHERAPY TO PEANUT-EFFICACY, SAFETY, AND ANTIBODY CHANGES

6.2.2.1 *The efficacy and safety of peanut OIT*

In peanut oral immunotherapy, 67% of the patients were desensitized, i.e. they could tolerate 1255 mg cumulative dose of peanut protein at the end-challenge. A larger proportion, 87%, was able to achieve the daily maintenance dose of 800 mg of peanut protein. A study from the UK reported similarly 62% of their patients passing the end-challenge.¹⁸⁶ When comparing the success rates of peanut oral immunotherapy, one must take into account differences in the published studies, thus inclusion criteria and protocols vary. In addition, published studies are based mostly on small patient populations, i.e. under 50 OIT patients. In the study by Varshney et al. in the USA, up to 84% (16/19) patients passed the end-challenge, but one patient was reported to experience mild symptoms and receive antihistamine.¹⁹⁷ A Japanese study reported 16/18 (89%) patients achieving the maintenance dose (3.5-7g of whole peanut), but no end-challenges were conducted.¹⁹¹ The rate of 89% is in line with our 87% with a very similar goal dose, four peanuts, which corresponds to approximately 3.2 g whole peanut.

Our secondary outcome in the study of peanut oral immunotherapy was the effect of the treatment on airways. Airway effects had not been studied during peanut oral immunotherapy before our study. Many peanut-allergic patients have asthma, as did the major proportion (69%) of our patients. None of our patients was excluded from the study due to poor asthma control. One patient

was diagnosed with asthma based on the baseline airway studies, but this patient received asthma treatment and was able to start immunotherapy. Uncontrolled asthma is a risk factor for fatal and near-fatal anaphylactic reactions in food allergies.³⁰ We studied the airways through two methods: Fractional exhaled nitric oxide, to show airway inflammation, and a methacholine bronchial challenge, to show bronchial hyperreactivity. In addition, we compared baseline spirometry results. We found no difference in airway measurements before and after the treatment, which indicates that with good asthma control throughout treatment, peanut oral immunotherapy does not cause bronchial hyperreactivity or airway inflammation.

Most of our patients (77%) reported some adverse effects during the eight-month build-up phase, which is similar to a bigger pooled study on children in peanut OIT that reported 80% of the study children experiencing side effects.
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No off-treatment period was conducted in our patients. In the published studies, off-treatment period results in a failure of desensitization in many patients,^{213,199} so in this regard any discontinuation of the treatment for research purposes may therefore not be justified as a routine approach.

Food allergy-related quality of life improved in our patients. In an Israeli study, patients with the worst quality of life improved during the food immunotherapy build-up phase, while those patients with better quality of life at baseline even decreased their quality of life.²⁵⁵ In our study, parents of the study children showed no change in the quality of life score. We must take into account that parents filled in the quality of life questionnaires at the clinic, and patients in many cases were accompanied by different parent on different visits.

We monitored specific IgE levels and found no statistically significant change after the build-up phase; in some patients, IgE decreased and in some it even increased. The duration of the build-up phase was eight months in our study protocol, and IgE levels may still increase in this phase until they start to decrease.^{189, 191} Immunotherapy patients are immunologically a heterogeneous group, as the course of their treatment provides patient-to-patient variations according to success in escalating the dose. In one-year samples, we found a decrease in IgE levels. In contrast to IgE, the specific IgG4 levels increased strongly already after the build-up phase, which is in accordance with previous studies.^{189, 191, 224}

6.2.2.2 Changes in sensitization profiles during peanut OIT

The microarray screening of IgE levels before and after the peanut OIT build-up phase showed that desensitization in peanut oral immunotherapy was highly allergen-specific. Specific IgE only to the major peanut allergens Ara h 2 and Ara h 6 decreased, but no effect was present on specific IgE to other nut seed storage proteins or on specific IgE to the peanut cross-reactive allergens,

the PR-10, LTP, and profilin families.

Peanut OIT had the strongest effect on the best markers of severe peanut allergy, i.e. Ara h 6 and Ara h 2.^{109, 256} These 2S albumin allergens share 59% amino acid sequence identity and are highly cross-reactive.¹¹⁵ The baseline specific IgE concentrations of Ara h 1 and Ara h 3 were lower, and the decrease after OIT failed to reach statistical significance. Our data showed also the dominance of a serological response to Ara h 2 over Ara h 1 and Ara h 3, which has been observed in some previous studies of peanut OIT.^{191, 199}

At baseline, sensitization to 2S albumins—other than peanut Ara h 2 and Ara h 6— was infrequent. Furthermore, peanut OIT had no effect on the low baseline specific IgE levels, and no novel sensitization to these 2S albumins occurred, either, which is in line with the fact that cross-reactivity is uncommon in the 2S albumin family.²⁵⁷ Specific IgE to the hazelnut and cashew 2S albumins was not affected by peanut OIT, which highlights the fact that peanut OIT modifies only peanut allergy despite concurrent allergy to other nuts.²⁵⁸

Our population was highly sensitized to PR-10 proteins. We included patients with moderate-to-severe peanut allergy, so patients were sensitized to the major peanut allergens in addition to the common co-sensitization to the minor allergen Ara h 8, which is associated with mild symptoms. Despite exposure to Ara h 8 in peanut flour and fresh whole peanuts, our patients exhibited no changes in the levels of IgE to Ara h 8, Bet v 1, or other PR-10 proteins. This might be due to the fact that Ara h 8 is degraded in the gastrointestinal tract, or because of the low content of Ara h 8 in the consumed products. Therefore, oral immunotherapy may be ineffective for IgE responses to Ara h 8. In contrast, in subcutaneous birch pollen immunotherapy, IgE to cross-reactive PR-10 proteins Aln g 1, Mal d 1, Cor a 1.0401, and Pru p 1 decreased.¹⁵⁵

Ara h 9 sensitization was rare in our population, which is in line with previous studies from Northern Europe.⁸² Sensitizations to other LTPs were also uncommon (5–17%) and mostly of a low level. OIT did not cause novel sensitizations for Ara h 9 or other LTPs. Ara h 9 content is low in peanut,⁸⁰ thus leading to the lack of an effect on Ara h 9-specific IgE.

We observed that peanut OIT increased the ratio of IgG4 to IgE antibodies for peanut-specific storage proteins Ara h 1, 2, 3, 6, and the whole peanut extract. Allergen-specific IgG4 antibodies emerge in the development of natural tolerance and during specific immunotherapy,^{14, 191} and in sensitized individuals, a high peanut-specific IgG4-to-IgE ratio predicts tolerance.^{147, 148}

In line with our study findings, with OIT, the ratio of peanut- specific IgG4-to-IgE can rise from ten- to even a thousand-fold.^{227, 148} In our study, the ratios for Ara h 6 and Ara h 2 increased most and exceeded the rise for whole peanut, Ara h 1, and Ara h 3. The low baseline rate of sensitization to Ara h 9 might explain why peanut OIT had no effect on the Ara h 9-specific IgG4-to-IgE ratio in our study. It has been argued that pretreatment IgE

response to a specific allergen may be needed for the induction of IgG4 antibodies.¹⁵⁴ However, the IgG4-to-IgE ratio for Ara h 8 remained unchanged, even though the majority of our patients were sensitized to Ara h 8 at baseline. The increase in IgG4 is in accordance with previous reports of decreasing IgE levels in ISAC, as IgG4 antibodies block IgE binding to the microarray.¹⁴⁹ The ISAC microarray has been even proposed to work as a tool to monitor the development of desensitization in immunotherapies to birch and timothy grass pollens,^{155, 154} and it could similarly benefit peanut OIT as a monitoring tool. As a clinical finding, we observed that the cumulative dose of peanut protein ingested during OIT was associated with the increase in the specific IgG4-to-IgE ratio. In patients with a higher cumulative allergen dose, increases in the IgG4-to-IgE ratios to whole peanut extract, Ara h 6, Ara h 2, and Ara h 3 were higher. A specific IgG4-to-IgE ratio might function as a proxy for success in OIT, as a higher IgG4-to-IgE ratio to peanut is associated with sustained unresponsiveness.¹⁹⁹

All of our patients had a moderate-to-severe peanut allergy and were sensitized to Ara h 2 and/or Ara h 6. In addition, the majority of the patients were also sensitized to Ara h 1 and/or Ara h 3, which indicates a more severe peanut allergy.¹⁰⁹ We were also able to analyze the change in IgE in relation to a broad spectrum of peanut-specific and cross-reactive allergens. Dividing the patient population into subgroups according to OIT success was not possible, though, due to the sample size.

7. CONCLUSIONS, CLINICAL IMPLICATIONS, AND FUTURE CONSIDERATIONS

Sensitization to birch pollen has a remarkable effect on sensitization to nut species, and the effect is strong both in southern and northern Finland. Our study results are generalizable outside Finland, for example Central Europe, which has less birch pollen than Helsinki region. The most strongly birch-cross-reactive species are hazelnut, almond, and peanut. Cashew and pistachio, and walnut and pecan cross-react the strongest. Despite the common finding of sensitization, many individuals with positive skin prick tests experience only mild symptoms restricted to the oral cavity. Knowledge of cross-reactivities allows more detailed patient advice, although further studies with food challenges are necessary. Our results imply that patients sensitized to some nuts may be able to receive more specific directions about which nuts can be consumed safely and which should be avoided. It would be possible to study cross-reactions in a smaller study population with in vitro studies, component-specific tests, and severity-graded nut challenges.

Many peanut-sensitized patients can reintroduce several other nut species into their diet, as species-specific sensitizations to other nut species are infrequent. However, care must be taken when consuming nuts, as species identification may be difficult, and in very sensitive patients even contamination may cause symptoms.

In diagnosing peanut allergy with the ISAC microarray, Ara h 2 and Ara h 6 are the best allergens. We do not recommend Ara h 8 for diagnosing peanut allergy. The levels of IgE to peanut seed storage proteins correlate with the challenge threshold dose. Use of this finding for an individual patient needs further assessment. A future study on the clinical relevance of the ISAC microarray for discriminating nut allergies other than peanut, e.g. Brazil nut, cashew, walnut, and sesame seed allergies, would be of interest and should include double-blind placebo-controlled nut challenges.

Oral immunotherapy is effective in desensitizing peanut-allergic children and adolescents. However, not all patients are able to increase the allergen dose and achieve desensitization. Severe reactions are possible even after longer periods of treatment, and so warning signs should be considered seriously. Further studies are needed to assess which patients benefit from the treatment the most. If particular attention is paid to asthma control, OIT does not have a harmful effect on bronchial hyperreactivity or airway inflammation. Larger studies are needed to confirm our results on airway inflammation and bronchial hyperreactivity. Long-term tolerance and safety, as well as any effects on

quality of life, should be assessed in longstanding studies. In addition, the efficacy of oral immunotherapy to peanut should be studied in different age groups and patients with very high risk of anaphylaxis. In future studies, quality of life in food allergy immunotherapy would be of great interest, as quality of life is an important factor in the overall benefits of a treatment. The treatment's effect on patients and families as a whole should be taken into account, and it is important to examine how life quality progresses in the longer term when the treatment continues or is discontinued due to a lack of motivation, or due to side effects.

No novel sensitizations emerge or previous sensitizations strengthen during oral peanut immunotherapy using a natural immunotherapy preparation. The treatment affects only the most important peanut seed storage proteins Ara h 2 and Ara h 6. The ratio of IgG4 to IgE to seed storage proteins increases during immunotherapy, and this increase is associated with the ingested cumulative peanut dose. The IgG4-to-IgE ratio might even serve as a measure for assessing the progress of immunotherapy. A larger study would offer more sensitivity for observing small changes in the sensitization profiles in IgE microarrays. Finally, validating specific IgG4-to-IgE ratio for the assessment of desensitization in OIT would require larger studies.

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